University of Alberta

INDIVIDUAL DIFFERENCES IN HYPOTHALAMIC-PITUITARY-ADRENAL FUNCTION IN SWINE: EXPERIENTIAL AND GENETIC FACTORS

by

SHELLEY ANN WEAVER ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

INDIVIDUAL DIFFERENCES IN HYPOTHALAMIC-PITUITARY-ADRENAL FUNCTION IN SWINE: EXPERIENTIAL AND GENETIC FACTORS

submitted by

SHELLEY ANN WEAVER

in partial fulfilment of the requirements for the degree of

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ABSTRACT

Individual differences in hypothalamic-pituitary-adrenal (HPA) function have been proposed to arise from experiential and genetic factors. The work described in this thesis has explored these differences with respect to early neonatal environment and the presence of the heritable mutation in skeletal ryanodine receptors known to cause Porcine Stress Syndrome (PSS).

Western blotting for the glucocorticoid receptor (GR) was developed in order to examine possible effects of treatments on this receptor in brain tissue. In pigs the highest level of GR expression was found in the frontal cortex followed by the hippocampus and hypothalamus.

The postulated mechanism for differential HPA function induced by neonatal handling in rats is via augmented central serotonin signalling during handling. Increasing serotonin early in life, via exogenous quipazine administration resulted in no change in central GR concentrations or glucocorticoid response to stressor applications in rats or pigs. As adults, neonatally quipazine injected rats had higher concentrations of 5-HT transporters in the paraventricular nuclei.

Pigs which had been subjected to neonatal handling were tested for HPA function. Neonatally handled pigs did not differ in GR concentrations in any brain region examined. As adults, handled pigs had higher plasma CBC and ACTH responses to stressors and lower free and total plasma cortisol concentrations during nadir basal sampling.

A comparison of pigs heterozygous for the mutated ryanodine receptor

and wild-type pigs showed no difference in GR receptor concentrations between genotypes. Heterozygous pigs had significantly lower basal nadir ACTH and cortisol concentrations with no difference in ACTH or cortisol responses to stressors.

In an extension of this work the expression of two calcium binding proteins and skeletal ryanodine receptors were examined in brain tissue from these two genotypes. Calreticulin concentrations did not differ between genotypes. Heterozygous pigs had lower calsequestrin concentrations in the hippocampus compared to wild-type pigs. Using *in situ* hybridization, skeletal ryanodine receptor mRNA was detected in the hippocampus.

The experiments presented in this thesis describe the development of animals which differ in HPA function resulting from early experiences. Differences in HPA function also arose from the heritable condition of PSS. Each situation resulted in a unique HPA profile thus emphasizing the complexity and flexibility of the development and function of the HPA.

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List of Abbreviations

ACTH	adrenocorticotrophin Hormone
ADX	adrenalectomized
ANS	autonomic nervous system
AP-2	activator protein-2
apo-sus	apomorphine-susceptible
apo-unsus	apomorphine-unsusceptible
AVP	arginine vasopressin
CBG	corticosteroid binding globulin
CBC	corticosteroid binding capacity
CR	calreticulin
CRH	corticotrophin releasing hormone
CS	calsequestrin
DET	detergent
ER	endoplasmic reticulum
5-HT	serotonin
5-HIAA	5-hydroxy indole acetic acid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamic-pituitary-adrenal axis
LVP	lysine vasopressin
MR	mineralocorticoid receptor

NGFI-A	nerve growth factor inducible factor A
PCR	polymerase chain reaction
PSS	porcine stress syndrome
PSE	pale soft exudative
PVN	paraventricular nucleus
SC	soluble cytosolic
SR	sarcoplasmic reticulum
sRyR	skeletal ryanodine receptor
T_3	triiodothyronine
T_4	thyroxine
VIP	vasoactive intestinal peptide
WCE	whole cell extract

INTRODUCTION

A major responsibility of research in the field of animal science is to attempt to provide answers to problems encountered in producing domestic animals. In swine production a current concern involves the deleterious effects of stress on animal performance in terms of feed intake, weight gains, disease susceptibility, and reproduction. In addition, society is also concerned about the welfare of animals in our modern production systems.

Studies involving the effects of stress on swine necessitates a clear understanding of the physiological systems involved in generating the stress response. Then and only then can one attempt to examine the effect of commercial production on the incidence of stress in pigs. A brief review of the research published on stress in pigs prompted the following statements

> "...we are sometimes over-hasty in interpreting results of experiments in terms of animal welfare when we are ignorant of much of the basic biology of the physiological and behavioral responses we are studying.... Given this degree of ignorance, how can we be confident of any statements about animal welfare based on such data?" (Rushen 1991).

Often only cursory analysis is performed on aspects of the stress response in pigs with hypotheses put forth to explain the final result. This area of research in pigs is also characterized by a lack of examination of the role of the brain in directing the stress responses. To date, the role of glucocorticoid receptors in the brain has not been examined in swine whilst numerous studies in rats have demonstrated that these receptors direct the degree of endocrine and often behavioural responses to stressors. Technically it is difficult to examine brain tissue from such a large animal and for certain aspects of function it is impossible. However, many aspects of neural responses can be examined and may provide clues as to the disparity within stress research results in swine.

A problem often encountered in stress research in swine is that of individual variation in responses. Rushen (1991) has cited "...alarming inconsistencies between studies" which have measured the stress response to the same stressor in different research units. Variation can even occur within individual facilities. Some of this variation can be attributed to individual variation in response to the stressor resulting from genetic or experiential differences. Individual differences can be associated with heritable changes in aspects of the stress response such as is the case for APO-sus (Rots et al. 1995) and Roman Low Avoidance rats (Walker et al. 1989). In rats, some individual differences in stress responses have been associated with early neonatal environment (Weaver and Meaney 1997) such as maternal deprivation (Rots et al. 1996), neonatal endotoxin exposure (Shanks and Meaney 1994), and neonatal handling (Meaney et al. 1993). In pigs, reduced variation has been reported within litters compared to across litters in adrenocortical response to exogenous adrenocorticotrophin hormone (ACTH) (von Borell and Ladewig 1992). Whether this reflects genetic, nutritional, or environmental effects is unknown.

Porcine Stress Syndrome is a known genetic difference in stress responses in pigs; however, the underlying mechanisms of hypothalamic-pituitary-adrenal (HPA) function disturbances are not known. Differences in adrenal sensitivity have been demonstrated (Hennessy 1986) but what drives these changes in adrenal morphology, which result in differential function, is not known. In addition, proposed differences in negative feedback are hypothesized (Geers et al. 1994) and yet sites for feedback have not been examined to date. To further complicate matters there are also discrepancies in the characterization of the stress response system for these animals in spite of decades of research.

The research described in this thesis seeks to address some of the issues cited above. Firstly, to examine neural glucocorticoid receptors and their relationship to HPA function in swine. Secondly, to analyze one aspect of the neonatal environment and its influence on stress responses, specifically that of neonatal handling and its proposed mechanism of augmented serotonin signalling. Lastly, to examine HPA function in pigs that are heterozygous for mutated ryanodine receptor, known to be the causative factor in Porcine Stress Syndrome. Chapter 2 describes the identification of neural glucocorticoid receptors in swine. In addition to identifying these receptors, regional distribution and developmental differences in glucocorticoid receptor concentrations in swine were also examined which are described in

Chapter 3. Whether augmentation of serotonin signalling in the neonate would increase glucocorticoid receptor concentrations in swine is described in Chapter 4. Chapter 5 is an extension of this work as the Quipazine treatment was also tested in rats. This treatment was hypothesized to dampen stress responses in adults and decrease serotonergic innervation of the hypothalamus in addition to augmenting hippocampal glucocorticoid receptor concentrations. In Chapter 6 the hypothesis that neonatal handling would effect differences in HPA function in swine was tested. The following two chapters focus on the relationship between HPA function and mutated skeletal ryanodine receptors. Chapter 7 describes the examination of whether differences exist between pigs heterozygous for mutated ryanodine receptor and normal pigs in basal or stress induced HPA function. In Chapter 8 the hypothese that skeletal ryanodine receptors are expressed in porcine brain and calcium binding protein concentrations would be upregulated by the presence of mutated skeletal ryanodine receptors were tested.

The objectives of the series of experiments reported in this thesis was to provide new and valuable information regarding the components of the HPA and factors involved in generating individual differences in HPA function in pigs.

CHAPTER 1

LITERATURE REVIEW

1.1. STRESS

The impact of stress on animals has become an important area of research due to the recognition that current animal husbandry systems can have deleterious effects on animal well-being as evidenced by the presence of abnormal behaviours, reproductive problems, high morbidity and mortality, as well as possible negative impacts on growth rate and feed conversion efficiency (Wiepkema 1988). In humans, stress has been linked to arterial disease, amenorrhea, impotency, hypertension, cardiovascular disease, headaches, ulcers, infectious diseases, cancer, and depression (Meaney et al. 1991a, Morse and Pollack 1988), further highlighting the importance of the impact of stress on animal and human health. Before it is possible to examine experimental parameters associated with stress, it is necessary to become familiar with the terminology used in this area of research.

1.1.1. Definitions

The term "stress" was first introduced in a physiological context by Selye in 1950 as "a state manifested by a specific syndrome which consists of all the non-specifically induced changes within a biologic system" (Asterita 1985). Selye further proposed a "General Adaptation Syndrome" and categorized the stages an organism progresses through during stress: alarm reaction, stage of resistance, and stage of exhaustion (Selye 1976). The alarm reaction occurs first and is characterized by immediate sympathoadrenomedullary discharge, followed by activation of the HPA which was labelled as the stage of resistance (Kopin et al. 1988). The last stage in the stress response is the stage of exhaustion which can proceed to death (Kopin et al. 1988). The General Adaptation Syndrome is characterized by the "diseases of adaptation": adrenal hypertrophy, gastrointestinal ulceration, and thymic and lymphoid shrinkage (Kopin et al. 1988). There are as many definitions of stress as there are books written on the subject; however, the definition found in Dorland's medical dictionary (1988) is quite thorough and acceptable:

> "The sum of biological reactions to any adverse stimulus, physical, mental, or emotional, internal or external, that tends to disturb the organisms homeostasis; should these compensating reactions be inadequate or inappropriate, they may lead to disorders."

Stressors are defined as "factors that activate specific physiologic mechanisms in the body" (Asterita 1985). Stressors can be physical, such as temperature and exercise, or psychological, such as anxiety, or grief (Selye 1976). Many stressors are both physical and psychological, as is the case for immobilization which contains an emotional escape reaction and a physical stressor of muscular work (Axelrod and Reisine 1984). The response to stressors differs and, therefore, the definition for stressor includes "specific physiologic mechanisms" (Axelrod and Reisine 1984, Ramsey 1982). The response involves the activation of three systems: firstly, behavioural, followed by the autonomic nervous system (ANS), and lastly, the neuroendocrine response (Moberg 1985).

1.1.2. The Stress Response

The stress response is elicited by these three interrelated systems which are activated in a specific intensity depending on the type of stressor, its intensity and duration, and the physiological and experiential status of the individual (Mason 1975). The stress response includes the combined action of these three systems.

The behavioral response to stressors involves avoidance of the stressor such as moving away from a dominant animal (Moberg 1985). Adjunctive behaviours occur when avoidance is not possible and this involves reactions such as chain nibbling by pigs on an intermittent feeding schedule which resulted in a lower increase in plasma cortisol concentrations (Dantzer and Mormede 1981). Humans have a larger array of possible behavioral responses in order to dampen the stress response such as denial, prayer, and intellectualization which resulted in a smaller increase in corticosteroid production (Sachar 1980). Many stressors cannot be avoided or denied and require more of a physiological response.

The sympathetic division of the ANS is involved in increasing energy availability for the "flight or fight" response coined by Cannon (Carlson 1992, Moberg 1985). Impulses from the spinal cord are relayed via the sympathetic preganglionic cholinergic neurons to the postganglionic fibres which release norepinephrine at their synapses on a variety of tissues (Kopin et al. 1988). Some of the results are increased heart rate, blood flow to skeletal muscle, and piloerection (Carlson 1992). There are also important synapses on the adrenal medulla which result in epinephrine release upon norepinephrine release from the sympathetic terminals (Kopin et al. 1988). The epinephrine induces a further excitation in addition to the effects of circulating norepinephrine by increasing cardiac output and vasodilation (Carlson 1992, Kopin et al. 1988). It is also involved in liberating glucose from glycogen stores in skeletal muscle (Carlson 1992). The ANS response occurs within seconds; for example, epinephrine concentrations peak within 1 minute and norepinephrine concentrations within 5 minutes after exposing rats to a noise stressor (De

Boer et al. 1988). This system is enlisted as the earliest stage of physiological defense when behavioral responses are not possible or sufficient.

The third, and possibly most complex, reaction is that of the neuroendocrine system. This system involves the communication between the central nervous system and the endocrine system and involves the hypothalamus and pituitary gland which not only produce their own endocrine secretions but also regulate other endocrine glands (Moberg 1985, Pelletier 1991). The final target in this cascade of reactions is the adrenal cortex which releases glucocorticoids in response to stressors (Moberg 1985). Cortisol, released into the plasma, promotes proteolysis of muscle, gluconeogensis from gluconeogenic amino acids and glycogen stores in the liver, decreased peripheral glucose use, lipolysis and the use of fatty acids for energy, ketogenesis, and immune suppression to prevent inflammation (Carlson 1992, Lehninger 1987, Meaney et al. 1991a). The effects of glucocorticoids result in increased substrates for the increased energy demands of the body as stimulated by the release of norepinephrine and epinephrine (Carlson 1992). The effects of glucocorticoids are necessary in that adrenalectomized rats are more susceptible to stress and may even die from a stressor that control rats can survive (Carlson 1992). The neuroendocrine system is characterized by a slower onset (relative to the ANS response) and a more sustained activation. The regulation of the neuroendocrine response involves numerous sites and hormones and is the focus of much of the research on stress.

1.1.3. Hypothalamic-Pituitary-Adrenal Axis (HPA)

The HPA neuroendocrine response to stressors involves the principal sites of the hypothalamus, pituitary gland, and adrenal cortex. Figure 1-1 (page 11) is a diagrammatic representation of the HPA. Activation of the HPA results in glucocorticoid release from the effector gland, the adrenal cortex. The glucocorticoids then target specific tissues primarily to mobilize energy reserves and suppress anabolic processes. The HPA activity is regulated in part by negative-feedback effects of glucocorticoids on further activation as well as neurotransmitters which communicate inhibition or activation upon the HPA.

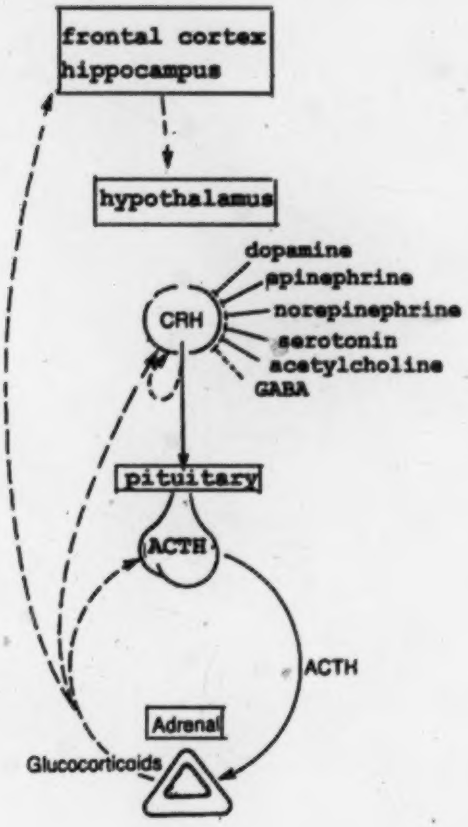
frontal cortex
hippocampus
hypothalamus
dopamine
epinephrine
norepinephrine
serotonin
acetylcholine
GABA
CRH
pituitary
ACTH
ACTH
Adrenal
Glucocorticoids

Figure 1-1. The hypothalamic-pituitary-adrenal axis (HPA). Exposure to stressors induces the release of CRH from the hypothalamic paraventricular nuclei. The peptides are transported to the anterior pituitary via the hypophysial portal system and stimulate ACTH release. ACTH then stimulates the release of glucocorticoids from the adrenal cortex. A negative-feedback loop exists whereby glucocorticoids inhibit further activation of the axis by acting on receptors at various sites within the HPA and at higher brain sites. Dashed-lines represent inhibitory effects and solid lines represent stimulatory effects (Johnson et al. 1992).

1.1.3.A. Hypothalamus

The hypothalamus is the site of synthesis of corticotropin-releasing hormone (CRH) which is the principal hormone used to convey the central perception of stress to non-neural tissues. CRH is synthesized in the parvocellular neurons of the paraventricular nucleus (PVN) whose axons project to the median eminence where CRH is released from the terminals into the capillaries of the hypophyseal portal vessels and transported to the anterior pituitary gland (Reisine 1985). CRH release is stimulated by dopamine, norepinephrine, epinephrine, acetylcholine and serotonin, whereas its release is inhibited by GABA, opioid peptide system, ACTH, and glucocorticoids (Johnson et al. 1992).

In the pituitary gland CRH stimulates adrenocorticotropin hormone (ACTH) release as well as synthesis of proopiomelanocortin, the ACTH precursor (Levin et al. 1989). CRH is found in many other areas of the brain and plays a role in behaviours such as anxiety, fear, and appetite (Morely and Levine 1982). In addition, a subset of PVN neurons project to the hindbrain where they release CRH following exposure to stressors. This stimulates arousal and sympathetic activation which further increases the response of the adrenal medulla to stress (Fisher 1989, Valentino et al. 1986).

1.1.3.B. Pituitary gland

Synthesis and release of ACTH into the blood stream occurs in a small group of corticotroph cells found in the anterior pituitary (2 to 3% of the total cell population) (Axelrod and Reisine 1984, Reisine 1985). In most species the most potent stimulator of ACTH release and synthesis is CRH, followed by arginine vasopressin (AVP) which acts alone as well as synergistically with CRH. Approximately 50% of CRH neurons also contain AVP as a co-secretagogue of CRH (Whitnall et al. 1985). In swine CRH is the most potent secretagogue of ACTH release followed by lysine vasopressin (LVP) which has an additive effect on CRH induced ACTH release and a synergistic effect on cortisol release at the adrenal gland (Abraham and Minton 1996, Minton and Parsons 1993, Janssens et al. 1995). LVP appears to have greater direct effects than exogenous CRH on the adrenal gland by eliciting greater cortisol responses following peripheral injection (Janssens et al. 1995). Release of ACTH is also stimulated by vasoactive intestinal polypeptide (VIP) and CCK-8 which are both found in the CRH-containing neurons of the PVN and act directly on the anterior pituitary (Axelrod and Reisine 1984, Reisine 1985). CRH-induced ACTH release is also potentiated by norepinephrine, epinephrine, oxytocin, and angiotensin II (Axelrod and Reisine 1984, Watanabe and Orth 1988). In swine, no synergistic effects of oxytocin or angiotensin II have been shown (Abraham and Minton 1996).

The release of ACTH is inhibited or reduced by a variety of central and peripheral molecules. Somatostatin, from the hypothalamus, blocks the stimulated release of ACTH by acting on many second messenger sites (Reisine 1985). High concentrations of somatostatin can desensitize its own receptor, therefore decreasing the effect of released somatostatin (Axelrod and Reisine 1984). Prolonged exposure to CRH results in a decrease in the number of CRH receptors on ACTH secreting cells and this effect is potentiated by VIP (Axelrod and Reisine 1984). This down regulation may not occur in primates, since continuous CRH infusion for 2 weeks (monkeys) or 24 hours (man) produced no decrease in elevated ACTH concentrations (Reisine 1985). Beta-adrenoreceptors also down regulate when high concentrations of epinephrine are present by decreasing the synthesis of new receptors (Axelrod and Reisine 1984). Release of ACTH from the pituitary is also inhibited by glucocorticoids functioning as negative-feedback regulators on further release of ACTH (Reisine 1985).

1.1.3.C. Adrenal cortex

ACTH stimulates glucocorticoid synthesis and release from the adrenal cortex and has a trophic effect on this structure (Johnson et al. 1992). The glucocorticoids, principally cortisol in primates and pigs and corticosterone in the rat, are synthesized in the adrenal cortex along with over 30 other steroids

(Jones et al. 1982, Lehninger 1987). The synthesis and release of glucocorticoids is stimulated by ACTH, which also increases the uptake and delivery of cholesterol and its metabolism to cortisol (Axelrod and Reisine 1984, Lehninger 1987, Winter 1992).

1.1.3.D. Corticosteroid binding globulin

Corticosteroid binding globulin (CBG) is a plasma glycoprotein synthesized by the hepatocytes of the liver (Hammond 1995). CBG binds circulating glucocorticoids with high affinity, therefore reducing their availability to target tissues (Hammond 1995). Plasma albumin will also bind steroids albeit with low affinity. It will therefore release bound glucocorticoids readily and, as such, it is not considered to affect bioavailability to a great extent (Westphal 1975). CBG is also found in the pituitary (not found in limbic structures such as the hippocampus) and binds glucocorticoids with high affinity and specificity and prevents their entry into cells, thereby acting as a buffer to glucocorticoid action (Dallman et al. 1987). CBG therefore protects organs from excess stimulation from circulating glucocorticoids at the same time as preventing their negative feedback modulation of ACTH release at the pituitary gland (Dallman et al. 1987, Meaney et al. 1985, Ratka et al. 1989). In rats and humans about 90% of circulating glucocorticoids are bound to CBG (Siiteri et al. 1982). In pigs, CBG binding of cortisol is one-fifth that for rats or humans in that only 20% of total cortisol is bound to CBG (Evans et al. 1988, Westphal 1975). There is no circadian rhythm in CBG concentrations in the pig (Barnett et al. 1981, Evans et al. 1988) unlike that found in humans and rats (Hammond 1995).

Numerous factors have been shown to influence CBG concentrations and they are often age and species specific. Chronic stress or chronic ACTH/corticosterone injections reduce plasma CBG concentrations (Armario et al. 1994, Neufeld et al. 1994, Spencer et al. 1996). Chronic stress has been shown to decrease (Kattesh et al. 1980) or have no effect on CBG binding capacity in pigs (Klemcke 1994). Acute stress also decreases CBG binding capacity in rats (Fleshner et al. 1995, Tannenbaum et al. 1997); however, this is dependent on the duration and severity of the stressor, with no effect on CBG found with short-term exposure to immobilization or exposure to mild stressors such as food deprivation (Marti et al. 1997) and novelty (exposure to an unfamiliar situation or object) (Tannenbaum et al. 1997). The converse is found in fetal animals in that increased glucocorticoids increased CBG concentrations (Treena et al. 1995). Maternal separation of neonatal rats induced a decrease in CBG concentrations (Meaney et al. in press 1998). In neonatal pigs CBG concentrations are significantly higher in low birth weight pigs (< or= 1.2 kg) (Klemcke et al. 1993) which may prevent tissue exposure to excess cortisol and, therefore, possible mobilization of limited energy stores.

1.1.3.E. Corticosteroid receptors

Glucocorticoid sensitive tissues contain cystosolic receptors which bind glucocorticoids and upon activation (conformational change in receptor structure induced by ligand binding) they are translocated to the nucleus where they bind to hormone response elements present on specific genes (Munck et al. 1984). These activated receptors then function to suppress or stimulate transcription (Munk et al. 1984). The receptors involved are the type I receptor or mineralocorticoid receptor (MR) which has high-affinity for glucocorticoids and aldosterone, and the type II receptor or glucocorticoid receptor (GR) which exhibits high binding affinity for the synthetic glucocorticoid analogs dexamethasone and RU 28362 (van Eekelen et al. 1988). MRs have a 10-fold higher affinity for corticosterone compared to GRs (Dallman et al. 1987, Reul and de Kloet 1985). In the rat brain, MRs are found in highest density in the lateral septum and the hippocampus (Chao et al. 1989, Dallman et al. 1987, Reul and de Kloet 1985). GRs are more widely distributed in the brain and are found in high concentrations in the lateral septum, nucleus tractus solitarius, amygdala, median eminence, paraventricular, supraoptic and arcuate nuclei of the hypothalamus, and at lower concentrations in the hippocampus (Chao et al. 1989, Dallman et al. 1987, Reul et al. 1987, Reul and de Kloet 1985, Sarrieau et al. 1988a). Within the hippocampus GRs are located in highest density in the CA1 and CA2 pyramidal neurons of Ammon's horn, with intermediate density in the dentate gyrus, and low concentrations in the CA3 and CA4 pyramidal cell subfields (Herman et al. 1989, van Eekelen et al. 1988).

Under basal plasma corticosterone concentrations in the rat (1.4 µg/dl) (circadian nadir), 80 to 90% of hippocampal MRs are occupied by corticosterone and during the circadian crest or after immobilization stress binding does not increase significantly (Dallman et al. 1987, Meaney et al. 1988, Ratka et al. 1989, Reul and de Kloet 1985, Reul et al. 1987). Hippocampal GRs are only 10% occupied at the circadian nadir and increase to 70 - 75% occupation during the circadian crest and at post-stress plasma corticosterone concentrations (Meaney et al. 1988, Reul and de Kloet 1985). It has been proposed that MR in the hippocampus are involved in the control of basal activity of the HPA axis and that GR are involved in the negative feedback effect of stress-induced elevations of glucocorticoids on ACTH release (van Eekelen et al. 1988).

1.1.3.F. Negative-feedback

Increases in plasma glucocorticoids have a negative feedback effect on further HPA activity by binding to GR receptors located in the pituitary, hypothalamus and higher brain sites (see Figure 1 page 11). Negative-feedback occurs at the hypothalamus to suppress CRH release and at the anterior pituitary gland to inhibit ACTH release (Keller-Wood and Dallman 1984). The inhibitory effect of glucocorticoids on ACTH release also acts indirectly via receptors in the medial-basal hypothalamus, hippocampus, and frontal cortex (Meaney et al. 1991a, Reisine 1985).

The hippocampus is an important site of the negative feedback action of glucocorticoids. Due to the presence of the highest concentration of corticosteroid receptors in the hippocampus, exogenous labelled corticosterone uptake and retention is greatest in the hippocampus with very little uptake by the hypothalamus or the pituitary (Dallman et al. 1987, Funder 1991, McEwen 1982). Dorsal hippocampectomy, or ablation of the hippocampus with kainic acid, resulted in a significantly lower suppression of plasma corticosterone in response to dexamethasone injection and hypersecretion of corticosterone post stress in rats (Herman et al. 1989, Feldman and Conforti 1980, Sapolsky et al. 1984). Brattleboro rats (congenitally deficient in vasopressin) have a depletion (24%) of GR only in the hippocampus and show a slower recovery from stressor-induced corticosterone elevations (Sapolsky et al. 1984). Aged rats also have a significantly lower hippocampal GR (30%) and it takes longer for plasma corticosterone concentrations to reach basal concentrations after stressor exposure (Sapolsky et al. 1984). Exogenous corticosterone given from days 29 to 34 reduced hippocampal GR by 40% in rats with no effect on concentrations in the septum, amygdala, hypothalamus, or pituitary, and these rats showed a significantly slower return to basal corticosterone concentrations after stressor exposure versus non-downregulated rats (Sapolsky et al. 1985). In addition, lactating rats which have elevated basal corticosterone concentrations, have significantly lower hippocampal GR concentrations (Meaney et al 1989b). The cell subfields CA1 and CA2 (highest GR) are the site of the major projection neurons of the hippocampus and via relays in the lateral septum and stria terminalis projections are sent to the PVN and medial basal hypothalamus (sites of CRH synthesis and release) (Herman et al. 1989). Higher concentrations of hippocampal GRs are associated with decreased hypothalamic CRH content and release (Herman et al. 1989, Liu et al. 1997, Plotsky and Meaney 1993, Viau et al. 1993).

1.2. HPA FUNCTION IN SWINE

Stress is a concern in commercial pig farms because of its effects on animal performance and the prevalence of stressors in conventional swine production systems. The use of stress responses as an indicator of decreased animal welfare (Barnett and Hutson 1987) brings stress issues in commercial animal production to the forefront of animal welfare concerns. Pathological consequences of prolonged exposure to stressors in swine include reproductive disorders in females such as delayed puberty, lack of behavioral receptivity, failure of ovulation or embryo implantation, spontaneous abortion, increased offspring mortality, decreased litter size, and poor conception rates (Varley 1991). Decreased growth rates and immunosuppression are seen in response to chronic stress (Barnett and Hutson 1987). Avoidance of stress responses in swine is next to impossible due to the variety of stressors present in swine barns such as chasing, cold, heat, novelty, transport, mixing of unfamiliar animals, restraint, (Barnett and Hutson 1987), feed restriction, crowding, weaning, mating, parturition, and aversive interactions with stock persons (Varley 1991).

1.2.1. Development of the Stress Response

The development of the HPA in neonatal swine has been examined; however, changes in CRH concentrations or the binding capacity of glucocorticoid receptors in the brain of neonatal pigs have not been tested. Plasma ACTH concentrations have also not been investigated; however, binding capacity of ACTH receptors in the adrenal glands were shown to be greatest at day 10 and to decrease significantly by day 24 (Klemcke and Pond 1991). Klemcke et al. (1993) demonstrated that adrenal response to ACTH did not change in pigs at 3 versus 7 days of age, with low birth weight pigs (less than 1.2 kg) having a significantly greater response at both ages compared to normal birth weight pigs. The ratio of adrenal weight to body weight was highest on the day of birth and then decreased for up to 60 days postpartum

(Dvorak 1972, Klemcke et al. 1993). Adrenal weight increased up to 2.5 fold as pigs aged from 3 to 31 or 60 days (Dvorak 1972, Klemcke and Pond 1991, Klemcke et al. 1993). Low birth weight female pigs had significantly greater adrenal weights relative to body weight at both days 3 and 7 postpartum (Klemcke et al. 1993) which may indicate a higher adrenal activity in these animals.

Maximum plasma total cortisol or free cortisol concentrations have been shown to occur on the day of birth (Kattesh et al. 1980). Plateau cortisol concentrations were achieved by day 5 (McCauley and Hartmann 1984). Kattesh et al. (1990) also showed total plasma cortisol decreased significantly after birth until a plateau by only day 3. Klemcke et al. (1993) found plasma cortisol concentrations were significantly lower on day 7 versus day 3 and the concentrations were significantly greater in female pigs of low birth weight. In three separate experiments which began blood sampling on day 3 *postpartum*, no significant difference was found in plasma total cortisol for up to 42 days *postpartum* (Kattesh et al. 1990, Klemcke and Pond 1991, Klemcke et al. 1992). In one experiment the percentage of unbound plasma cortisol was found to decrease linearly over the 42 days *postpartum* (Kattesh et al. 1990).

CBG bound cortisol was highest at birth and decreased to a nadir by day 3 after which it increased until day 42; however, the differences were not significant (Kattesh et al. 1990). Plasma CBG concentrations have been found to increase significantly over days 7 to 42 *postpartum* and these changes are postulated to be responsible for the decrease in free plasma cortisol concentrations that occurred over the same time period (Kattesh and Roberts 1993).

The plasma cortisol concentrations in neonatal pigs can be summarized from the literature as being highest at birth, after which the periparturant increase is completed within a few days *postpartum* and then remains unchanged until up to 42 days of age. The level of free cortisol in the plasma decreases over this time period as a result of the increasing CBG concentrations. Concurrently total adrenal weights are increasing; however, insufficient data are available for adrenal responsiveness to ACTH.

The response of neonatal pigs to stressors has been tested using overnight (16 hour) maternal deprivation (Klemcke and Pond 1991). Maternal deprivation resulted in a 3 fold increase in plasma total cortisol; these authors therefore proposed that pigs aged 3 to 31 days are capable of responding to stressors and as a result they do not show a stress hyporesponsive period like that found in neonatal rats (Klemcke and Pond 1991). Rat pups maternally deprived for 2 hours had significant increases in corticosterone when tested at day 5, 10, 15 or 20, with no significant increase seen at day 3 however at all time points the increase is significantly lower than that found in adult rats (Kuhn et al. 1990). The 2 week postnatal stress hyporesponsive period seen in rats is considered a "relative" response, with significant, although not dramatic, responses detected to a variety of stressors (Kuhn et al. 1990) The two different conclusions from very similar results for swine and rats is due to the lack of a numerical definition for stress hyporesponsive period, with conclusions based on differences relative to adults.

Weaned pigs have been shown to have increased plasma cortisol from stressors such as mixing (Dybkjaer 1992), weaning (Funderburke and Seerley 1990, Worsaae and Schmidt 1980), chasing (Worsaae and Schmidt 1980), 16 hour maternal deprivation (Klemcke and Pond 1991), and increased plasma ACTH in response to a hypoxia stressor but no ACTH increase due to pentobarbital anaesthesia (Moss et al. 1990). It can be summarized that pigs have the ability to respond to stressors early in life and that they are exposed to a variety of stressors during commercial production.

1.2.2. Basal HPA activity

Cortisol release in pigs is governed by a circadian rhythm with crests detected at 0700 h and nadirs at 1900 h when blood sampling was carried out at 6 hour intervals (Klemcke et al. 1989). Hourly blood sampling demonstrated lower plasma cortisol concentrations from 1200 to 2400 h and higher concentrations from 2400 to 1200 h (Griffith and Minton 1991). Blood sampling at 10 minute intervals between 0800 and 1600 h revealed a steep rise in corticosteroids that began at 0900 h, peaked within 1 hour and then declined by midday, with afternoon values consistently lower (Barnett et al. 1981).

ACTH concentrations in the plasma peak at 0700 h and the nadir occurs at 1900 h with sampling at 6 hour intervals (Klemcke et al. 1989) high values between 2400 and 1200 h and low values between 1200 and 2400 h (Griffith and Minton 1991). The rhythm of ACTH secretion does not parallel that of cortisol as evidenced by the low correlation coefficient (=0.10) between the two hormones (Griffith and Minton 1991). However, Klemcke et al. (1989) found a higher correlation between the two (r=0.34) and found that a linear increase in cortisol resulted from a $\log_{10}$ increase in ACTH.

1.2.3. Individual Differences in HPA responsivity

von Borell and Ladewig(1992) have shown that the variation in response to exogenous ACTH, ambulation and vocalization response to an open field test, and behaviour in the home pen, is lower within litters of pigs than between them and have postulated that excitability is genetically determined, established early in life, or both. von Borell and Hurnik (1991) reported on the cortisol response to exogenous ACTH in sows exhibiting high levels of stereotypic behaviour compared to those with low levels of stereotypic behaviour and found stereotypic sows had a significantly greater response to ACTH. The researchers proposed that under chronic stress there is a genetic difference revealed between sows in adrenocortical reactivity, in that the increased cortisol is an adaptive mechanism to fulfil the increased energy demands of the more physically active sows exhibiting high levels of stereotypies (von Borell and Hurnik 1991). In Beilharz and Cox (1967) the heritability estimate for differences in ambulation in an open field test was determined to be low (0.16), suggesting little influence of genetics.

Pearce et al. (1989) pooled data on cross-sectional area of the adrenal cortex and found a significant negative correlation with growth rate and postulated that differences were due to differences in the dominance hierarchy. Cortisol is produced by the clear cells of the zona fasciculata, and the compact cells of the zona reticularis, of the adrenal cortex, and both areas are regulated by ACTH (Tyrrell et al. 1991). Excesses and deficiencies of ACTH alter both structure and function of the zona reticularis and the zona fasciculata, with excess ACTH resulting in hyperplasia and hypertrophy of both layers (Tyrrell et al. 1991). As a result, animals that are exposed to a regular fixed dose of ACTH show increased glucocorticoid responses to the dose over time, primarily as a result of adrenal hypertrophy (Johnson et al. 1992). Pigs that show a hyper-responsiveness to adrenal stimulation upon exogenous ACTH administration, with no difference in clearance rate of cortisol, have decreased cytoplasmic area of the zona fasciculata cells (Zhang et al. 1987). Chronic ACTH stimulation resulted in depletion of the lipid stores of the clear cells of the zona fasciculata which are a store of cholesterol used for cortisol synthesis (Tyrrell et al. 1991). Chronic ACTH exposure may result in the structural and functional changes seen in pigs that are hyper-responsive to exogenous ACTH. Zhang et al. (1990) reported that pigs classified as high responders to ACTH injection given exogenous human CRH had no significant difference in ACTH response but cortisol responses were significantly greater, indicating hypersensitivity of the adrenal cortex. The development of a hypersensitive cortex may be a purely genetically determined difference; however, the correlation between adrenocortical sensitivity to ACTH and behavioral differences in OFT performance (von Borell and Ladewig 1992), along with the low heritability estimates for activity in OFT, suggest other factors may determine this physiological differences.

Pigs of the same age, sex, and genetic origin often show differences in response to ACTH injection, with some pigs showing a higher concentration of peak cortisol but no difference in the time to reach peak or decline to basal, or in metabolic clearance rate (Hennessy 1986). In these pigs the zona fasiculata cells of the adrenal cortex secrete less cortisol per cell due to decreased cytoplasmic area, and as a result possible decreased precursor cholesterol stores; however, they have a greater adrenocortical cell mass (Zhang et al. 1987). Both situations emphasize a different stress response compared to normal controls.

Pigs handled unpleasantly (pigs given a brief shock upon approaching the stockperson) after weaning have been reported to have significantly higher basal free and total corticosteroid concentrations (Hemsworth et al. 1981; 1987) or a trend towards higher corticosteroid concentrations (Paterson and Pearce 1992) compared to pigs handled pleasantly (allowing pigs to approach the stockperson and interact with no aversive signals given from the stockperson). The presence of a human for two minutes resulted in a significant increase in plasma corticosteroid concentrations in unpleasantly handled pigs, with no acute response seen in pleasantly handled pigs (Hemsworth et al. 1981; 1987), or greater although not significant increases in unpleasantly handled pigs (Hemsworth and Barnett 1991, Hemsworth et al. 1986). It has been proposed that unpleasantly handled swine experience a chronic stress response based on the greater basal plasma corticosteroid concentrations (Hemsworth et al. 1981; 1987), higher plasma total protein and glucose, and lower plasma urea concentrations (Barnett et al. 1983). However, the cross-sectional area of the adrenal cortex of such animals did not differ (no cortical hypertrophy) (Paterson and Pearce 1989). In addition, the adrenal response to exogenous ACTH also did not differ between pleasantly and unpleasantly handled pigs (Hemsworth and Barnett 1991). Rushen (1991) has reported that changes in discrete secretory episodes of cortisol may be more indicative of a chronic stress response rather than mean basal values and that the adrenal response to ACTH may also be a questionable measure of chronic stress. It has not yet been proven that unpleasantly handled pigs are experiencing chronic stress but they do show a significantly greater acute stress response to the presence of a human.

It can be concluded that pigs can differ in HPA function and that the origin of these differences may be genetically or environmentally determined. The neonatal period may represent a sensitive period of development for the influences of the environment on future HPA function. This has been shown to be the case in rats (Meaney et al. 1985) but as yet is unexplored in pigs.

1.3. NEONATAL HANDLING

The technique of handling neonatal rats was first investigated in the 1950's. Sporadic reports on the phenomenon continued up into the 1970's but it was not until the mid 1980's that a concentrated effort to determine the mechanisms involved occurred. It is now known which hormones, neurotransmitters, receptors, and second messengers are involved in causing the attenuation of the stress response in rats handled early in life (Meaney et al. 1989a; 1991b; 1992).

1.3.1. Effects of Neonatal Handling

Experiments performed in the late fifties and sixties revealed an effect of experimenter handling of neonatal rats on their stress response, and behaviour (Ader and Grota 1969, Hess et al. 1969, Levine 1957; 1961, Levine et al. 1967). The handling involved the experimenter holding the rat pups or removing and isolating the pups from the litter and the dam, or electrically shocking the pups, starting from the first day postnatally and continuing once per day for 21 days (until weaning) for a period of either 3 or 15 minutes per treatment (Ader and Grota 1969, Hess et al. 1969, Levine 1957; 1961, Levine et al. 1967). Handled rats in Levine's first experiment (1957) weighed significantly more (7%) at weaning and at day 70 than rat pups not handled postnatally. In this experiment a physiological stress (20% glucose solution injected I.P.) applied at day 80 resulted in a significant difference in the left adrenal gland weights: nonhandled rats had heavier adrenals which possibly indicated a greater secretion of ACTH acting on the adrenals (Levine 1957). In 1961 Levine reported that handled rats had a greater and more rapid corticosterone response to acute electric shock than nonhandled rats, which is contradictory to subsequent research on stress responses in handled rats. Hess et al. (1969) examined the response of handled and nonhandled rats to the stressor of exposure to a novel environment for 3 minutes and found that handled rats had a significantly lower increase in corticosterone and a faster return to basal concentrations compared to nonhandled rats. Levine (1967) also tested the response of handled rats to a novel environment and found significantly lower elevations in corticosterone in handled rats. Ader and Grota (1969) exposed handled and nonhandled rats to a novel environment for 5 seconds or 3 minutes in an attempt to explain the results of Levine (1961) of greater corticosterone secretion due to acute stressors. Again handled rats had a significantly lower elevation in corticosterone with no difference in response due to the length of exposure (Ader and Grota 1969).

More recently, research has confirmed that handled rats responded to stressors with a lower corticosterone and ACTH elevation and a faster return to basal plasma concentrations (Meaney et al. 1985; 1989a, Weinberg et al. 1978). Rats handled in infancy and exposed to a rat hole board (equally spaced holes in the floor of a cage with or without objects present which represents a novelty stressor) had lower elevations in plasma corticosterone 15 minutes after a 3 minute exposure than nonhandled rats (Weinberg et al. 1978). A 30 minute exposure to vibratory stress (cage placed in shaker bath) resulted in handled rats having a significantly lower corticosterone concentration at 0, 30, 60, or 90 minutes after exposure to the stressor and a faster return to basal corticosterone concentrations (by 90 minutes) compared to nonhandled rats in which corticosterone concentrations failed to return to baseline concentrations by the end of the experiment (Meaney et al. 1985; 1991b). In rats exposed to a restraint stressor for 20 minutes, handled rats had significantly lower plasma corticosterone and ACTH elevations and the absolute decline to baseline concentrations was faster emphasizing a faster rate of recovery irrespective of the magnitude of increase (Meaney et al. 1989a).

1.3.2. Changes in the HPA Axis

The effects of postnatal handling in rats resulted in a decrease in corticosterone and behavioral responses to stress. The handling process affected the HPA axis in some way which resulted in rats relatively hyporesponsive to stressors. Research has been directed at determining which point on the HPA axis handling had its effect.

1.3.2.A. Adrenal Gland

At the level of the adrenal gland, the possibility existed that handled rats hyposecreted corticosterone at all times compared to nonhandled. In comparisons of basal plasma corticosterone concentrations, there were no significant differences between handled and nonhandled rats (Meaney et al. 1989a; 1991b; 1992). There was also no difference in the circadian pattern of corticosterone plasma concentrations, with peak concentrations found at 20:00 for handled and nonhandled rats with sampling 6 times over 24 hours

(Meaney et al. 1989a; 1992). The metabolism of corticosterone has also been examined using injections of labelled corticosterone which resulted in no significant difference in the half-life of corticosterone between handled and nonhandled rats (Grota 1976). At doses of labelled corticosterone that mimic plasma concentrations occurring under restraint stress, no difference in corticosterone clearance was detected between handled and nonhandled rats (Meaney et al. 1989a). Rats handled postnatally did not have significantly more CBG than nonhandled rats (Meaney et al. 1992).

As the effect of handling does not appear to be due to changes in basal corticosterone, its clearance rate, or binding to CBG, the next possible site to examine was the release of corticosterone from the adrenal gland as stimulated by pituitary ACTH. As was the case for corticosterone, no differences were found in basal ACTH plasma concentrations between nonhandled and handled rats (Meaney et al. 1989a). However, the elevation of ACTH after a restraint stressor was significantly lower in handled rats (Meaney et al. 1989a). *In vitro* application of ACTH to adrenal tissue from handled and nonhandled rats resulted in no difference in corticosterone release (Grota 1976). Exogenous ACTH application also showed no difference in the elevation in plasma corticosterone (Meaney et al. 1992).

1.3.2.B. Pituitary and Hypothalamus

Since differences in the response of the adrenal to ACTH did not occur between handled and nonhandled rats and no differences were found in basal plasma corticosterone concentrations or its metabolism, the next site in the HPA was tested: the pituitary gland with its release of ACTH as stimulated by CRH. Since ACTH elevations are lower in stressed handled rats it may be that the anterior pituitary has a lesser response to CRH secreted from cell bodies in the paraventricular nucleus of the hypothalamus (Dallman et al. 1987). Extracts of the median eminence (site of CRH release into portal circulation) from open field stressed handled rats injected into the carotid artery of dexamethasone-blocked assay rats resulted in a lower plasma corticosterone elevation than from handled rats, demonstrating a lower hypothalamic CRH release in handled rats (Zarrow et al. 1972). Exogenous ovine CRH caused increases in plasma corticosterone; however, there were no significant differences between handled and nonhandled rats (Meaney et al. 1989a). It can be concluded that although there was no difference in pituitary sensitivity to CRH in handled versus nonhandled rats, CRH release from the hypothalamus was lower during stress in handled rats. In fact, postnatal handling does result in significantly reduced basal concentrations of immunoreactive CRH, arginine vasopressin, and CRH mRNA in the median eminence, and significantly reduced depletion of CRH upon restraint stress, thus indicating a decreased synthesis and release of CRH (Plotsky and Meaney 1993, Viau et al. 1993). Since adrenal glands and the pituitary do not respond differently to stimulation from ACTH and CRH, respectively, and hypothalamic CRH and pituitary ACTH are reduced by the action of negative feedback, the next question was whether the site of altered tissue sensitivity would be neural tissues containing glucocorticoid receptors responsible for negative feedback.

1.3.2.C. Negative-feedback sites and GR

Exogenous administration of either corticosterone or dexamethasone suppressed plasma ACTH responses to stress more effectively in handled than in nonhandled animals (Meaney et al 1989a). The mean dose required to provide 50% suppression of plasma ACTH for both steroids is five to 10 times lower in handled than nonhandled rats (Meaney et al. 1989a). Adrenalectomy, which removed the glucocorticoid negative-feedback signal, eliminated the differences in plasma ACTH responses to restraint stress between groups (Viau et al. 1993). Administration of low levels of corticosterone replaced the differences in adrenalectomy-induced ACTH hypersecretion during stress between handled and nonhandled rats, providing further evidence for the greater sensitivity of the handled rats to the negative-feedback effects of circulating corticosterone (Viau et al. 1993). Handled rats had significantly greater (30 to 40%) [illegible] concentrations (as measured by increased binding capacity and immunoreactive GR concentrations) in the hippocampus compared to nonhandled rats but no significant differences in MR or GR concentrations in the hypothalamus, pituitary, septum, or amygdala (Meaney and Aitken 1985, Meaney et al. 1985; 1985b; 1989a; 1991b). GR mRNA concentrations are significantly increased in the hippocampus of handled rats throughout all cell subfields, indicating increased synthesis of GR (O'Donnell et al. 1994). Meaney (et al.1989a) treated adult handled rats for 5 days with corticosterone and allowed 2 days for steroid clearance. In the handled rats that had subsequently received corticosterone, hippocampal GR density was down-regulated to concentrations that were indistinguishable from those of nonhandled rats, with no downregulation of GR occurring in the hypothalamus or pituitary. This treatment also eliminated the differences in stress responses between handled and nonhandled rats (Meaney et al. 1989a). Thus the increased density of hippocampal GR in handled rats mediated the differential HPA responsivity between handled and nonhandled rats (Meaney et al. 1989a).

1.3.3. Thyroid Hormones and Serotonin

Subcutaneous injections of triiodothyronine (T_3) and thyroxine (T_4) in rats on days 1 and 2 postnatally resulted in a significant increase in hippocampal GR but no significant differences in GR concentrations in the pituitary gland or hypothalamus measured at 90 days of age (Meaney et al.

1987). Hypothyroidism induced by propylthiouracilL (a thyroid hormone synthesis inhibitor) reversed the effects of handling on hippocampal GR concentrations, as well as reducing hippocampal GR concentrations in nonhandled rats with no effect on pituitary or hypothalamic concentrations (Meaney et al. 1987). Applications of thyroid hormones to hippocampal cell cultures had no effect on the binding capacity of GR and therefore the effects of T_3 and T_4 are not directly on hippocampal cells (Mitchell et al. 1990a).

Injections of T_3 in adult rats two hours before decapitation resulted in a significant increase (27%) in 5-hydroxy indole acetic acid (5-HIAA:5-HT) ratio in the hippocampus (Mitchell et al. 1990b). In addition, handled rat pups killed on postnatal day 7 had significantly greater 5-HIAA:5-HT ratios in the hippocampus compared to nonhandled pups (Mitchell et al. 1990b). The effect of thyroid hormones on hippocampal GR was therefore hypothesized to be via the direct effects of 5-HT (Mitchell et al 1990b). The hippocampus receives serotonergic inputs primarily from the median raphe nucleus and also from the dorsal raphe nucleus (Segal 1989). The hippocampus also contains some of the largest density of 5-HT receptors and is especially rich in 5-HT_{1a} receptors in the dentate gyrus and CA1 pyramidal cell field (Segal 1989). Serotonin applied to hippocampal (predominantly neuronal) cell cultures caused a dose dependent increase in binding capacity (the maximal number of binding sites) of GR ,with no effect on binding affinity (ligand concentration where 50% of binding sites are occupied) or on MR (Mitchell et al. 1990a). The maximum effect on receptors was seen at 10 nM 5-HT and increased receptor concentrations were still present up to 40 days after 5-HT removal from the medium (Mitchell ~~et al.~~ 1992). Exposure must be for a minimum of 4 days while no further increases were seen after day 14 of 5-HT application (Mitchell et al. 1990a). Ketanserin (a 5-HT$_2$ receptor antagonist) blocked the effect of 5-HT on GR binding capacity and the 5-HT$_2$ receptor agonists 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane and m-trifluoromethylphenylpiprazine mimicked the results of 5-HT but to a lesser extent (Mitchell et al. 1990a). *In vivo* experiments showed ketanserin injections from day 2 to 14 postnatally blocked the effect of handling on hippocampal GR concentrations and reduced them in nonhandled animals compared to nonhandled non-treated controls (Mitchell et al. 1990b). Injections on days 1 and 2 postnatally of 5,7-dihydroxytryptamine (serotonergic neurotoxin) reduced hippocampal 5-HT concentrations by 80% and significantly decreased GR binding capacity in the hippocampus (Mitchell et al. 1990b). It has been proposed that the effects of 5-HT are on 5-HT$_2$ receptors; however, more recently studies have shown that the 5-HT effect on GR concentrations in hippocampal cell cultures is mediated by the recently cloned 5-HT$_7$ receptor subtype (Laplante et al. 1996). In embryonic hippocampal cell culture, 50 nM of the 5-HT$_7$ receptor agonist 5-carboxamidotryptamine increases GR expression to a level identical to that induced by 5-HT (Laplante et al. 1996).

The intracellular effect of 5-HT binding is on cAMP concentrations which were significantly increased in cultured hippocampal cells exposed to 10 Nm 5-HT for 4 days with no effect on cGMP concentrations (Mitchell et al. 1992). Ketanserin application also blocked the 5-HT-induced increase in cAMP while quipazine (a non-selective 5-HT agonist) increased cAMP concentrations to a similar extent to that seen with 5-HT (Mitchell et al. 1992). Cultures exposed to 8-bromo cAMP (cAMP analog) for 4 days had increased GR binding capacity to a similar extent as application of 5-HT (185% versus 191%, respectively) (Mitchell et al. 1992). Experiments with rat pups have shown an identical effect of handling on cAMP concentrations to that found in hippocampal cultures with 5-HT (Francis et al. 1996). Rat pups handled for the first 7 days of life and then sacrificed immediately after handling on day 7 showed an 8 to 10-fold increase in hippocampal cAMP concentrations compared with nonhandled pups (Francis et al. 1996). Examination of activated adenylyl cyclase using [^{3}H] forskolin autoradiography also showed an increase in binding in all subfields of the hippocampus (Francis et al. 1996). The effects of handling on hippocampal cAMP concentrations were blocked by the concurrent administration of either ketanserin or propylthiouracil, as was the case for the effect of handling on GR expression (Francis et al. 1996).

The effects of handling on forebrain GR concentrations appear to involve the activation of ascending 5-HT input into the hippocampus. This effect appears to depend upon the pituitary-thyroid system. The 5-HT effect is then mediated by activation of a 5-HT$_7$ receptor which is positively coupled to cAMP.

1.3.4. cAMP-inducible transcription factors

Altered rates of cAMP formation are known to affect gene transcription (Delmas et al. 1993, Francis et al. 1996). These cAMP effects appear, in part, to be mediated by a range of cAMP-responsive transcription factors and their interactions with specific DNA binding sites. Recent studies have shown that hippocampal expression of at least two such factors, activator protein-2 (AP-2) and nerve growth factor inducible factor A (NGFI-A also known as zif268 or krox24) are altered by early handling (Francis et al. 1996). In contrast, there were no effects on CREB, phosphorylated CREB, cFos, cJun, or NGFI-B (Francis et al. 1996). In addition, handling effects on transcription factors were mediated by both thyroid hormone and 5-HT systems (Francis et al. 1996).

In 7 day old rat pups handling produced significantly increased NGFI-A mRNA expression (8-10 fold), as detected with *in situ* hybridization, in all cell fields of the hippocampus. The effect on NGFI-A mRNA expression was evident immediately after handling and persisted for up to 240 minutes following handling (Francis et al. 1996). The increased NGFI-A mRNA was paralleled by an increased NGFI-A immunoreactivity (Francis et al. 1996). Importantly, the handling effect on NGFI-A immunoreactivity was blocked by co-administration of either propylthiouracil or ketanserin (Francis et al. 1996). This again supported the original work showing the involvement of the thyroid hormones and their subsequent effect on 5-HT in producing the handling-induced upregulation of GR.

Handling produced a similar effect on AP-2 expression (Francis et al. 1996). AP-2 mRNA concentrations were increased uniformly across the hippocampus in response to postnatal handling (Francis et al. 1996). The increased mRNA concentrations were again paralleled by changes at the protein level (Francis et al. 1996) and this handling-induced increase in AP-2 immunoreactivity was also blocked by simultaneous administration of either propylthiouracil or ketanserin (Francis et al. 1996).

To date, the mechanisms involved in the effect of neonatal handling in rats have been partially determined. The effect of neonatal handling in other species has not been examined in spite of its dramatic effects on HPA function.

1.4. HANDLING IN SWINE

Research on the effects of handling on pigs have generally involved adult animals and have been concerned with the differential effects of pleasant versus unpleasant handling on production variables and physiological correlates (Hemsworth et al. 1981; 1987, Paterson and Pearce 1992). There has been some preliminary work on the effects of neonatal handling on the behaviour of swine (Hemsworth and Barnett 1992, Hemsworth et al. 1986b) but in general this is a novel area.

1.4.1. Neonatal Handling

Hemsworth et al. (1986b; Hemsworth and Barnett 1992) have tested the response of swine to neonatal handling treatments. In one experiment pigs were either artificially reared, left on the sow and handled once per day for 27 days, or once per day 5 times per week for the next four weeks following weaning, compared to nonhandled control pigs (Hemsworth et al. 1986b). Handling treatments involved introducing pigs to a human who sits in a pen and allows pigs to approach, and attempts to stroke them, with no aversive contacts with the animals for 2 minutes per day (Barnett and Hemsworth 1986). Artificially reared pigs had significantly greater ambulation rates in an open field test at 29 weeks of age, with handled and nonhandled pigs not differing significantly (Hemsworth et al. 1986b). Artificially reared and handled pigs both approached and interacted with a human faster, and remained within 0.5 m of the experimenter longer than nonhandled pigs when tested at 6 times over 10 to 24 weeks of age (Hemsworth et al. 1986b). The authors have proposed that a sensitive period exists for socialization in the pig and that it occurs at some time before 8 weeks of age (Hemsworth et al. 1986b). The second experiment performed by this group tested handling over four different postnatal time periods: 0 to 3, 3 to 6, 6 to 9, and 9 to 12 weeks of age versus a nonhandled control group and found that handling applied at 0 to 3 or 9 to 12 weeks of age significantly reduced the fear response to humans when tested at 18 weeks of age (Hemsworth and Barnett 1992). It has been proposed that 0 to 3 weeks of age encompasses the critical period, since the effects seen at 9 to 12 weeks old may be due solely to the recent exposure of the pigs to humans and therefore reflects a habituation response to humans, (Hemsworth and Barnett 1992). When these animals were tested again at 20, 22, or 24 weeks of age no significant differences were found in their behavioral response to humans possibly due to experience with humans after the handling treatment application (Hemsworth and Barnett 1992). Hemsworth and Barnett (1992) also reported that the variation in behavioral response to humans increased from 18 to 24 weeks, which supports the supposition that post-treatment interaction with humans with some pigs confounded the behavioral response of the pigs to humans when tested at 20 to 24 weeks of age. The presence of a human cannot be considered a stressor if habituation has occurred subsequent to treatments and it is therefore possible that behavioral differences may have been detected if an appropriate stressor was applied. It may also be that a 2 minute individual handling treatment involving stroking if the piglet approached the experimenter would not produce permanent changes in behaviour due to insufficient and inconsistent stimulation. If the HPA changes that occur in rats could be evoked in pigs one can see how this handling treatment would be insufficient due to variations in interactions between the experimenter and the animal, whereas in the handling treatment in rats the pups are simply removed from the mother for 15 minutes per day with no specific handling treatments applied.

Neonatal handling in pigs does appear to produce permanent behavioral changes, however, HPA function in such animals has not been examined to date. In addition, as explained above, the behavioral test used was confounded by the treatment. The results also indicate that an environmentally sensitive period exists in pigs during the first three weeks of life. It can be concluded that environmentally sensitive periods in development may exist in the pig but that their involvement in determining HPA function throughout life has remained relatively unexplored.

1.5. PORCINE STRESS SYNDROME

An established difference in HPA function in pigs results from a mutation in the skeletal muscle ryanodine receptor which functions as a sarcoplasmic and endoplasmic reticulum calcium channel. Pigs carrying this mutation exhibit Porcine Stress Syndrome and alterations in HPA function.

1.5.1 Porcine Stress syndrome and Malignant Hyperthermia

Malignant hyperthermia is a condition which develops in genetically predisposed individuals upon exposure to halogenated anaesthetics (Lister 1987). The syndrome is characterized by skeletal muscle rigidity, hypermetabolism, increased respiratory rate, systemic acidosis, and a rise in body temperature (Lister 1987, MacLennan and Phillips 1992). Irreversible shock and anoxia develops leading finally to cardiac arrest (Hails 1978, Lister 19857). This condition occurs in swine as well as humans and in swine it is referred to as the Porcine Stress Syndrome (PSS) (Lister 1987, MacLennan and Phillips 1992). Upon slaughter, pigs with this condition exhibit an accelerated rigor mortis, resulting from a rapid decrease in intramuscular pH while the carcass temperature is still high (Lister 1987). This results in denaturation of muscle proteins and resultant water loss from the tissues which gives the meat a pale soft exudative appearance (PSE) and a reduced commercial value

(Lister 1987). The PSS syndrome has been shown to result from a single nucleotide substitution in the gene encoding the ryanodine receptor (RyR) (Fujii et al. 1991) and this identical mutation is found in a subset of human malignant hyperthermia cases (Fletcher et al. 1995). In a study which tested 3,275 pigs from Canadian farms, the incidence of carriers for this syndrome (Nn) was 19% and homozygous animals (nn) was 0.7% emphasizing the predominance of carriers rather than homozygous animals in the commercial swine industry (O'Brien et al. 1993).

1.5.2 Ryanodine receptors

The RyR is a calcium channel which can be labelled with the plant alkaloid ryanodine and can be activated by caffeine (MacLennan and Phillips 1992). In skeletal muscle, RyR is a 565 kDa homotetramer which connects the sarcoplasmic reticulum (SR) with the transverse tubule membrane (T-tubule) which surrounds each myofibril (Barchi 1994). Activation of these receptors allows the passage of calcium ions (Ca^{2+}) from the SR into the myofibrils, thereby initiating muscle contraction (Barchi 1994). PSS in pigs has been attributed to a replacement of cytosine with thymine at nucleotide 1843 in the skeletal muscle RyR (sRyR) gene (Fujii et al. 1991). This mutation results in the replacement of arginine at position 615 with cysteine (Fujii et al. 1991). Fujii et al. (1991) demonstrated that the skeletal RyR in PSS pigs exhibited channel opening at lower concentrations of ATP, Ca^{2+} and caffeine, and dampened inhibition of opening by Mg^{2+}, and Ca^{2+} compared to normal pigs.

In the brain the function of the RyR is not known; however, it is postulated to amplify the Ca^{2+} signal that initiates from ligand-gated Ca^{2+} channels during depolarization by releasing intracellular Ca^{2+} stores (Furuichi et al. 1994). Furuichi et al. (1994) have demonstrated the presence of skeletal (s), cardiac (c), and brain (b) isoforms of RyR widely distributed throughout the rabbit brain using *in situ* hybridization with type-specific probes. Regions of note were the hippocampus, which expressed all 3 forms of the RyR with sRyR uniformly expressed in pyramidal CA1-CA4 cells and granule cells of the dentate gyrus, and the higher levels of bRyR in CA1 subfields compared to other levels of the hippocampus (Furuichi et al. 1994). RyR binding capacity has been shown to be 3.7 fold greater in hippocampal mossy fiber synaptosomes than in cortical synaptosomes and was localized to presynaptic terminals in rats (Padua et al. 1996). McPherson and Campbell (1990) have reported high concentrations of RyR in the hippocampus using a sRyR polyclonal antibody. In the avian brain RyR exists in the dendritic shaft, and cell body, and axons, and is absent in the presynaptic boutons or synaptic spines with no correlation to specific neurotransmitter systems (Ouyang et al. 1993, Walton et al. 1991). The presence of sRyR in porcine brain has not been examined.

1.5.3 HPA function

Stress-susceptible swine have been reported to have higher basal plasma cortisol (Marple et al. 1974, Schaefer et al. 1990) or lower basal cortisol (Mitchell and Heffron 1981). Marple et al. (1974) has reported that stress-susceptible swine have higher CBG binding capacity and lower affinity for cortisol. By contrast Nyberg et al. (1988) found no difference in CBG binding capacity between homozygous, carrier, or wild-type pigs. Stress-susceptible pigs also exhibited a lower corticoid release upon injection of ACTH (Sebranek 1973), indicating adrenal insensitivity to ACTH.

Marple et al. (1972) reported that stress-susceptible pigs exhibited 3 fold higher a.m. basal ACTH concentrations, with no difference in plasma corticoids. When stressed with fluctuating temperatures a.m. ACTH concentrations increased to a greater level in stress-susceptible pigs than normal pigs with no significant difference in corticoid concentrations between groups or between control and stress (Marple et al. 1972). Pigs of Nn genotype had a significantly greater ACTH response to a blood sampling stressor than NN pigs (Roberts et al. 1996). Nine week old Nn pigs exhibited highest cortisol followed by NN with lowest concentrations in nn animals after halothane testing (Nyberg et al 1988). This would reflect stressor-induced cortisol concentrations. At 12 weeks of age cortisol concentrations were not different across genotypes but after transport nn had the highest cortisol concentrations, followed by NN and Nn, with the only significant difference being between Nn and nn (Nyberg et al. 1988). Prior to transport but after an anaesthesia stressor, nn and Nn pigs had lower concentrations of cortisol compared to NN pigs (Geers et al. 1994). After transport stress and a second anaesthesia stressor, nn pigs showed no difference in cortisol while Nn and NN pigs showed significant reductions in plasma cortisol compared to the original stressor (Geers et al. 1994). The nn pigs were at a significantly higher cortisol level after transport compared to being lowest prior to transport compared to Nn and NN pigs (Geers et al. 1994). There are a number of problems with this study that makes interpretation of the data difficult. Firstly, there are no pre-stress or basal cortisol concentrations, making it difficult to assess the strength of changes in response to stressors. In addition, the handling and injection of anaesthetics would represent a stressor and, therefore, the experiment may actually reflect the response to repeated stressors. One point was that nn pigs had no change in cortisol pre- or post-transport and again adrenal insensitivity may be responsible with large changes occurring in ACTH.

Stress-susceptible pigs have been shown to exhibit impaired negative feedback of cortisol demonstrated by a lack of dexamethasone suppression of plasma ACTH (Sebranek 1973). Geers et al. (1994) have proposed possible differences in negative feedback effects of cortisol on ACTH release as being responsible for differences between genotypes (Geers et al. 1994), as Nn and

NN animals showed a reduction in plasma cortisol after transport with no difference occurring in nn pigs. This is possible, as is adaptation to the repeated anaesthesia stressor. Either scenario is impossible to elucidate without basal cortisol and ACTH concentrations.

In conclusion, earlier studies have demonstrated impaired negative feedback of cortisol, and adrenal insensitivity, in PSS pigs ,with more recent studies demonstrating a lack of dramatic differences in plasma cortisol in response to stressors in PSS pigs. In no instance has a complete HPA profile been generated with pigs genotyped to distinguish heterozygous from homozygous halothane responders. Without an understanding of basal HPA function, differences in stress responses between normal and PSS pigs are difficult to ascertain.

1.5.4. Calreticulin

The presence of mutated sRyR leads one to hypothesize that this mutation may result in compensatory changes in calcium binding proteins. One such protein, calreticulin, has been shown to be involved in functionality of GR and may play a role in generating differences in HPA function between genotypes.

Calreticulin is a calcium binding protein composed of 418 amino acids with a predicted M_r of 46,567 Da and a (reduced) M_r on SDS-PAGE gels of 55

Kda (Fliegel et al. 1989, Smith and Koch 1989). Calreticulin is composed of three domains namely N, P, and C with high affinity Ca^{2+} binding of 0.6-1 mol/mol and a disassociation constant of 10 μM in the proline rich P-domain and low affinity (2 mM) high capacity (18 mol/mol) Ca^{2+} binding in the carboxy terminal or C-domain and no calcium binding in the N-domain (Baksh and Michalak 1991). It contains a KDEL endoplasmic reticulum retention signal and a possible nuclear localization signal (Fleiegel et al. 1989, Rojiani et al. 1991) with no transmembrane sequences (Fliegel et al. 1989). Calreticulin has been identified in liver, uterine smooth muscle, and brain using polyclonal antibodies (Milner et al. 1991, Treves et al. 1990). mRNA for calreticulin (1.9 kb) was detected in liver, uterine smooth muscle, brain, kidney, heart, and skeletal muscle (Fliegel et al. 1989, Milner et al. 1991). Calreticulin has been proposed to function as a Ca^{2+} storage protein in non-muscle cells and in fact *in vitro* cultures of muscle cells that are overloaded with Ca^{2+} in medium show increased calreticulin synthesis and therefore indicate a possible role for calreticulin in sequestering Ca^{2+} (Fliegel et al 1989).

More recently, Burns et al. (1994) have demonstrated a role of calreticulin in inhibiting binding of the GR to glucocorticoid response elements (GRE) of genes due to similarities in the amino acid sequence of the GR DNA binding region (K_xFFKR) and calreticulin binding sequence (KL_xFFKR). Using gel mobility shift assays they have demonstrated inhibited

GRE binding of glutathione S-transferase/ GR DNA-binding region fusion proteins by 5 µg of calreticulin (Burns et al. 1994). The N-domain of calreticulin was shown to be responsible for the inhibition in DNA binding (Burns et al. 1994). In addition, reporter gene assays using mouse L fibroblasts co-transfected with GRE luciferase reporter plasmid and calreticulin expression vector (PSVL-CRT) resulted in an 85% decrease in dexamethasone-induced increase in luciferase activity (Burns et al. 1994). Transfection with sense orientated calreticulin cDNA decreased GR sensitive cytochrome P450 gene mRNA and protein in mouse L cells (Burns et al. 1994). In addition, calreticulin has been shown to impair glucocorticoid-sensitive tyrosine aminotransferase gene expression in the McA-RH7777 hepatocyte cell line without interfering with cAMP-induced expression of this gene, thus demonstrating its specific interaction with GR (Burns et al. 1997).

It can be concluded that calreticulin has both roles in Ca^{2+} sequestering and modulating steroid receptor interactions with steroid-responsive elements of genes. Whether the presence of mutated sRyR leads to changes in the expression of calreticulin is unknown.

1.6. CONCLUDING STATEMENTS

The objectives of this thesis are to examine individual differences in HPA function in swine with respect to early environment, specifically the effects of exogenous 5-HT and neonatal handling, and the genetically determined differences generated by mutated sRyR in PSS pigs. The literature reviewed has described the HPA predominantly in relation to rats, as much of this axis remains unexplored in pigs. In addition, a description of the development of the HPA in pigs has been provided along with examples of individual differences in HPA function in pigs. A review of neonatal handling in rats described the mechanisms involved and the results from two experiments in pigs on neonatal handling have been reported. The section on PSS in pigs described this heritable mutation and the research, to date, on HPA differences resulting from this mutation. A description of calreticulin and its role in GR function provides a possible mechanistic link between PSS and altered HPA function.

The first two experimental chapters of this thesis address the detection, distribution, and developmental changes in brain GR in pigs and technical aspects of its measurement. The following chapter describes an analysis of exogenous 5-HT administration in neonatal pigs on brain GR concentrations. In the next section (Chapter 5) the effects of neonatal 5-HT signalling augmentation on HPA function and GR concentrations in rats was examined.

The effects of neonatal handling on HPA function in pigs is described in Chapter 6 followed by an analysis of differences in HPA function found in PSS pigs in Chapter 7. The final experimental chapter examined the presence of sRyR in the hippocampus and its effects on calcium-binding proteins.

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CHAPTER 2

IDENTIFICATION OF GLUCOCORTICOID RECEPTORS IN PORCINE BRAIN

2.1. INTRODUCTION

GR has been examined in porcine adipose (Chen et al. 1995), lung (Yarney et al. 1990), and thymus tissue (Weatherill and Bell 1984) using receptor binding assays. Problems arise with the conventional receptor binding assays in that receptors bound to endogenous ligand that have been translocated to the nucleus are not detectable. In addition, receptors in forms which are unable to bind ligand are also undetectable (Bresnick et al. 1989). In order for GR to be able to bind glucocorticoids it must be in a correct conformational form which is induced by the association of GR with heat shock proteins 90, 56, and 70, as well as p23 protein (Pratt 1993). Upon binding of glucocorticoids this complex of proteins dissociates from GR, thus rendering the receptor capable of translocating to the nucleus (Pratt 1993). Upon recycling of GR back to the cytosol the receptor is unable to bind ligand until the protein complex is added, resulting in an altered conformation which exposes the ligand binding site (Pratt 1993). The inability of receptor binding assays to detect all forms of GR necessitates adrenalectomizing animals 12-16 hours prior to sacrifice in order to clear endogenous hormones

(McEwen et al. 1974). Adrenalectomized (ADX) rats maintained for longer than 18 hours exhibit upregulated GR which can confound experimental results (McEwen et al. 1974). In previous work we have shown that western blotting provides a more accurate measure of GR protein concentrations than binding assays, which do not detect unoccupied receptors that are not bound to heat shock protein complexes or nuclear GRs (O'Donnell et al. 1995).

Marks et al. (1986) examined porcine GR from liver using western blotting and found steroid binding, immunoreactivity, and DNA binding are conserved between rats and pigs. To our knowledge, western blotting to examine GR expression has not been performed on porcine brain tissue. This series of experiments examined the immunological detection of GR in specific brain regions in neonatal male pigs using different polyclonal (rabbit), anti-human, glucocorticoid receptor antibodies.

2.2. METHODS

2.2.1. Animals

All experiments were performed under the approval and guidelines of the Canadian Council on Animal Care. Experiments involving pigs used 4 male 14 day old Pig Improvement Canada pigs. Pigs were anaesthetized with 40 mg/kg Ketamine HCl i.m. and decapitated upon confirmation of anaesthesia. The pituitary gland was collected and the frontal cortex, hypothalamus, and hippocampus were dissected out on ice within 3 minutes of decapitation. Tissue samples were snap frozen in liquid nitrogen and stored at -72^0 C until analysis.

Six male Long-Evans, hooded rats (Charles River, St-Constant, Quebec) weighing 140-180 g were used for the ADX study with 3 used as sham surgery controls. Neonatal rats used were 7 day old Long-Evans female pups which were used for the experiment reported in Chapter 5 (section 5.2.1.). The study on rat strain used adult male rats (200 - 250 g) from three strains: Long-Evans hooded, Fisher, and Sprague-Dawley. All rats were decapitated unanesthetized, the pituitary gland was collected and the frontal cortex, hypothalamus, and hippocampus were dissected out on ice within 3 minutes of decapitation. Tissue samples were snap frozen in liquid nitrogen and stored at -72^0 C until analysis.

2.2.2. Adrenalectomies

Bilateral adrenalectomies were performed on 3 rats under Metofane anaesthesia and sham surgeries (bilateral incisions above the adrenal glands) were carried out on a further 3 rats. All animals were provided with 0.9% (w/v) saline as drinking water and five days after surgery unanesthetized rats were sacrificed using decapitation in the morning. The hippocampus was dissected out on ice within 3 minutes of decapitation. Tissue samples were snap frozen in liquid nitrogen and stored at -80^0 C until analysis.

2.2.3. Sample preparation

Tissues were homogenized on ice, using a polytron homogenizer, in 3 volumes of ice cold TEDGM buffer (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate, pH 7.4) containing aprotinin (1µl/ml) and phenylmethylsulfonyl fluoride (PMSF) (10µl/ml). Rat tissue was disrupted using a Vibra Cell sonicator. The homogenates were centrifuged at 100,000g for 45 minutes at 4°C in a Beckman Ultracentrifuge. The supernatant, which represented a soluble cytosolic fraction, was collected on ice and frozen at -72^0 C. Protein concentrations were determined using the Bradford protein assay reagent from Bio-Rad (Bradford 1976).

2.2.4. Western blotting

Samples containing equivalent amounts of protein were electrophoretically separated in a Novex minigel apparatus (Helixx Technologies) using discontinuous 6% (w/v) or 8% (w/v) Tris-glycine SDS-PAGE gels. Recombinant human GR (Affinity BioReagents, N.J., U.S.A.) was used as a control for antibody detection. The samples were boiled for 3 minutes in equal volumes of 2X sample buffer (0.13M Tris-HCl, 4% (w/v) SDS, 0.005% (w/v) bromophenol blue, 20% (w/v)glycerol, and 5% (v/v) ß-mercaptoethanol). Proteins were transferred to nitrocellulose membranes using the Novex minigel semi-dry transfer apparatus (Helixx Technologies) with Towbin transfer buffer (Towbin et al. 1979) containing 0.03% (w/v) SDS. Membranes were dried for 30 minutes at room temperature and blocked with 5% (w/v) skim milk powder. The primary GR antibodies were polyclonal, (rabbit) anti-human, glucocorticoid receptor antibodies #57 and #51 from Affinity BioReagents, N.J., U.S.A. A ß GR isoform (present in human tissue (Oakley and Cidlowski 1996)) specific polyclonal (rabbit) antibody from Affinity Bioreagents was also tested. Antibodies were titrated against a constant level of protein and optimal dilutions (specific signal in the absence of non-specific bands and general membrane background) of the primary antibody were incubated with the membrane overnight at 4^0C. The membranes were washed with 4 changes of TBST (20 mM tris base, 140 mM NaCl and 0.01% Tween 20) after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody was incubated with the membrane for 1 hour at room temperature. Both antibodies were diluted with TBST containing 0.5% (w/v) skim milk powder. Hyperfilms (Amersham) were exposed to chemiluminescence via a horseradish peroxidase-linked secondary antibody and the ECL detection system (Enhanced Chemi-Luminescence) (Amersham Life Sciences). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent loading amongst neural tissue samples. A suitable control for comparing non-neural tissue (pituitary gland) with neural tissue is not known. All western blots were performed in at least duplicate.

2.2.5. Affinity Labelling of GR

A porcine frontal cortex sample was powderized in liquid nitrogen using a pestle and mortar and an aliquot of the powderized tissue was homogenized in TEDGM (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate) adjusted to pH 8.3. Cytosolic soluble fractions were obtained and protein concentrations were determined using the Bradford protein assay reagent from Bio-Rad (Bradford 1976). GRs were covalently labelled by incubating 400 µg of protein with 100 nM

[^{3}H]dexamethasone mesylate (specific activity of 35.5 Ci/mmol) at 4^{0}C overnight. Additional incubations were performed with 100 nM [^{3}H]dexamethasone mesylate plus a 100 fold excess of cold RU28362 (specific GR agonist) or a 1000 fold excess of cold dexamethasone in order to determine specificity of binding. Unbound steroid was removed by mixing the binding reaction with an equal volume of dextran coated charcoal (0.1% (w/v) dextran and 1% (w/v) charcoal in TEDGM) and centrifuging at 10,000g for 1 minute to remove unbound steroid. The samples were then concentrated with Centricon 30 filters (size cutoff of 30 kDa) by centrifuging at 4^{0}C for 30 minutes at 2,200 RPM. A 30 µl volume of concentrated samples were electrophoretically separated on a 6% (w/v) PAGE gel along with ^{14}C unstained molecular weight markers. In order to amplify the [^{3}H]dexamethasone mesylate signal the gel was impregnated with the scintillant 2,5-diphenyloxazole (PPO). The gel was dehydrated in DMSO (dimethyl sulfoxide) and then incubated with 22% (w/v) PPO in DMSO for 60 minutes at room temperature. The gel was washed repeatedly with room temperature distilled water until no strong odour of DMSO remained and loose crystals of PPO had been removed. The gel was then dried using a vacuum gel dryer at 65^{0}C and exposed to pre-flashed Kodak X-OMAT R film at -70^{0}C for 14 days. Molecular weights for the GR bands for affinity labelling and western blots were calculated using [^{14}C] unstained molecular weight markers and the Molecular Analyst^{TM}\PC program (Bio-Rad). The curve was fitted using a four-parameter logistic model where parameters are estimated by nonlinear least-squares curve fitting. Intensity of band signals for all results were quantified using relative optical densitometry with the Bio-Rad image analysis system.

2.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the Super Anova Statsview software package from Abacus Concepts. Data presentation is in the form of means with standard errors of the means. Statistical analyses on regional differences in the intensity of the two bands in the doublet were performed using ANOVA followed by Student-Newman-Keuls *post hoc* tests. Unpaired Students t test was used to test the signal intensity of hippocampal GR between ADX and sham operated rats.

2.4. RESULTS

2.4.1. Banding pattern with #57 GR antibody

Figure 2-1 demonstrates immunoreactive protein species obtained with the #57 GR antibody in all tissues examined (panels A-D) with a predominant doublet found in each case. The doublet appeared as two bands with calculated relative mobilities of 95 and 87 kDa. A nitrocellulose membrane containing transferred proteins was also blotted with only the secondary antibody as a control and no bands were detected (data not shown). We also examined tissue collected from a stillborn baby pig left overnight in the barn and Figure 2-2 shows that in this case only the upper 95 kDa band remained. A comparison of hippocampal tissue from adult rats and neonatal pigs revealed the presence of a doublet in the rat hippocampus, although not as distinguishable than that found in pigs (Figure 2-3A). Neonatal rats show an identical banding pattern in hippocampal GR to neonatal pigs (Figure 2-3B). An examination of immunoreactive GR across four brain regions amongst three stains of adult rats showed differential doublet expression depending on the strain of rat (Figure 2-4).

2.4.2. Identity of 95 kDa band

Figure 2-5 shows that only the lower band in porcine frontal cortex is specifically labelled with [^{3}H]dexamethasone mesylate which corresponds to the 87 kDa band. The upper 95 kDa band did not bind the GR ligand as evidenced by no signal above the 87 kDa band. Figure 2-6 shows the results of titrating the ß GR specific antibody against a constant amount of protein from all four brain regions of porcine brain tissue with only nonspecific signals at the 66 kDa range and no specific signal at 95 kDa. In fact, figure 2-7 shows the presence of an approximately 66 kDa band that was also detected upon titration of one of the lots of the #57 GR antibody and this band appeared occasionally while performing western blotting with other lot numbers.

When densitometric optical density values from the films were analyzed for each of the bands found in the doublet, only those for the 87 kDa band significantly differed ($p < 0.0001$) across brain region, whilst those for the upper band were not significantly different (Figure 2-8B).

2.4.3. GR detection using #51 antibody

Figure 2-9 shows the banding pattern obtained with the #51 GR antibody, in porcine frontal cortex tissue, upon titration of the antibody against 50 µg of protein. A single 87 kDa band was detected along with a relatively slower migrating band detected for the recombinant human GR which was also the case for the #51 antibody (see Figure 2-7). In order to confirm that the band was GR, hippocampal tissue from ADX and sham-operated rats were analyzed using western blotting with the #51 antibody (Figure 2-10). A significant ($p< 0.05$) upregulation of immunoreactive GR in the hippocampus of ADX rats was detected.

C

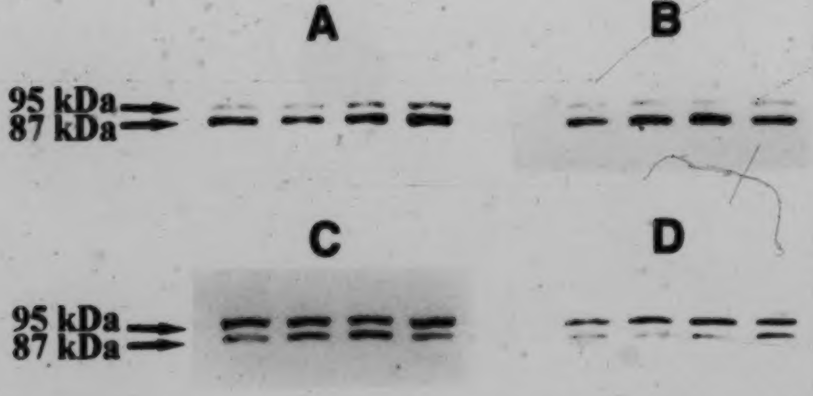

Figure 2-1. Immunoreactive GR banding pattern in individual brain regions. Tissue samples from 4 animals were electrophoretically separated on 8% (w/v) SDS-PAGE gels with one brain region per gel. Panel A pituitary (50µg protein), Panel B hypothalamus (30µg protein), Panel C hippocampus (50µg protein), Panel D frontal cortex (70µg protein). The antibody used was the #57 anti-GR antibody.

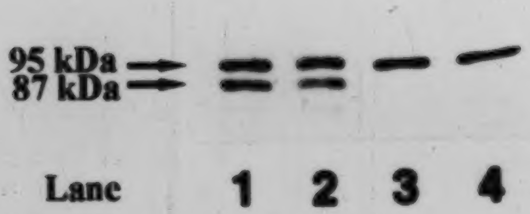

Figure 2-2. The effect of post-mortem duration prior to tissue collection on immunoreactive GR banding pattern. Western blot for hippocampal tissue from 2 animals collected under experimental conditions (lanes 1 and 2). Lanes 3 and 4 contains GR from hippocampal tissue collected more than 8 hours after death from 1 animal and frozen at -20⁰C prior to analysis. Each lane was loaded with 30µg of protein and separated on an 8% SDS-PAGE gel. The antibody used was the #57 anti-GR antibody.

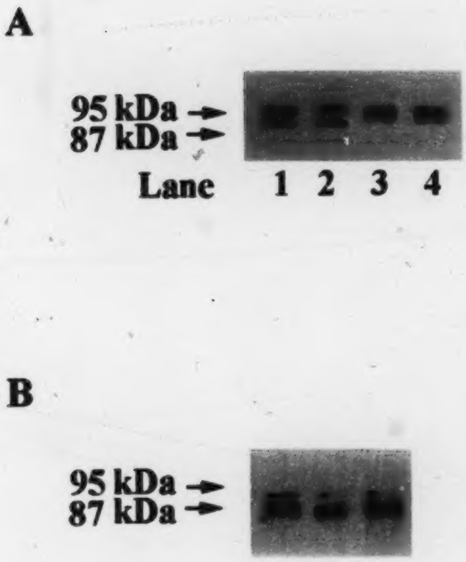

Figure 2-3. Species differences in immunoreactive GR banding pattern. A: western blot for hippocampal tissue from 2 neonatal male pigs and 2 adult male rats. Lanes 1 and 2 are porcine samples and lanes 3 and 4 are rat samples with equivalent protein loaded in each lane. B: western blot for hippocampal tissue from 3 female rat pups sacrificed on day 7. The antibody used was the #57 anti-GR antibody.

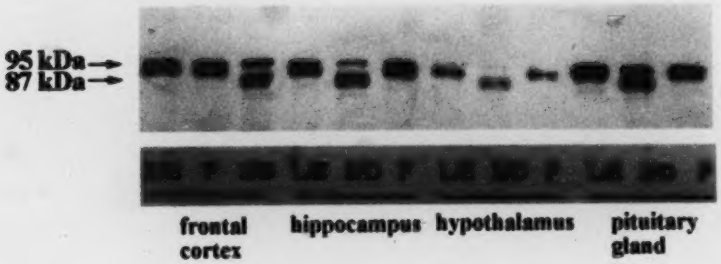
95 kDa
87 kDa
LE
F
SD
LE
SD
F
LE
SD
F
LE
SD
F
frontal cortex
hippocampus
hypothalamus
pituitary gland

Figure 2-4. Effect of rat strain on immunoreactive GR banding pattern. Western blot for four brain regions from 3 adult male rats from three different strains: Long-Evans (LE), Fisher (F), Sprague-Dawley (SD). The antibody used was the #57 anti-GR antibody.

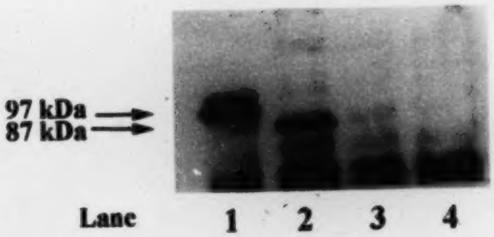

Figure 2-5. Labelling of GR with $[^{3}\mathrm{H}]$dexamethasone mesylate in porcine frontal cortex. Lane 1 shows the $[^{14}\mathrm{C}]$ labelled unstained molecular weight marker for 97 kDa. Lane 2 is sample incubated with 100 nM $[^{3}\mathrm{H}]$dexamethasone mesylate, lane 3 is in the presence of 1000 fold excess cold dexamethasone, and lane 4 with 100 fold excess RU28362.

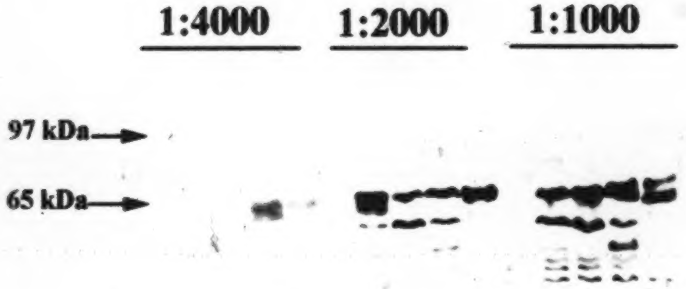

Figure 2-6. Titration of ß GR antibody in porcine brain tissue from 4 sites. Three dilutions of primary ß GR antibody (1:4,000, 1:2,000, and 1:1,000) incubated with 4 brain sites (from left to right for each dilution: pituitary gland, frontal cortex, hypothalamus, and hippocampus) from one pig with a constant amount of protein loaded per lane.

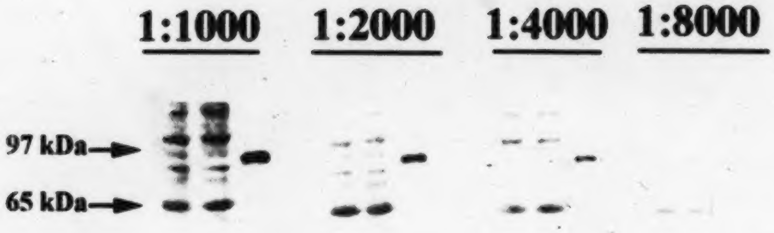
1:1000
1:2000
1:4000
1:8000
97 kDa
65 kDa

Figure 2-7. Titration of #57 GR antibody in porcine frontal cortex. Lot number 019-118 #57 GR antibody incubated with 2 lanes of frontal cortex tissue from the same pig (the first 2 lanes from left to right) and recombinant human GR (third lane). Four antibody dilutions were used (1:1,1000, 1:2,000, 1, 4000, 1:8,000).

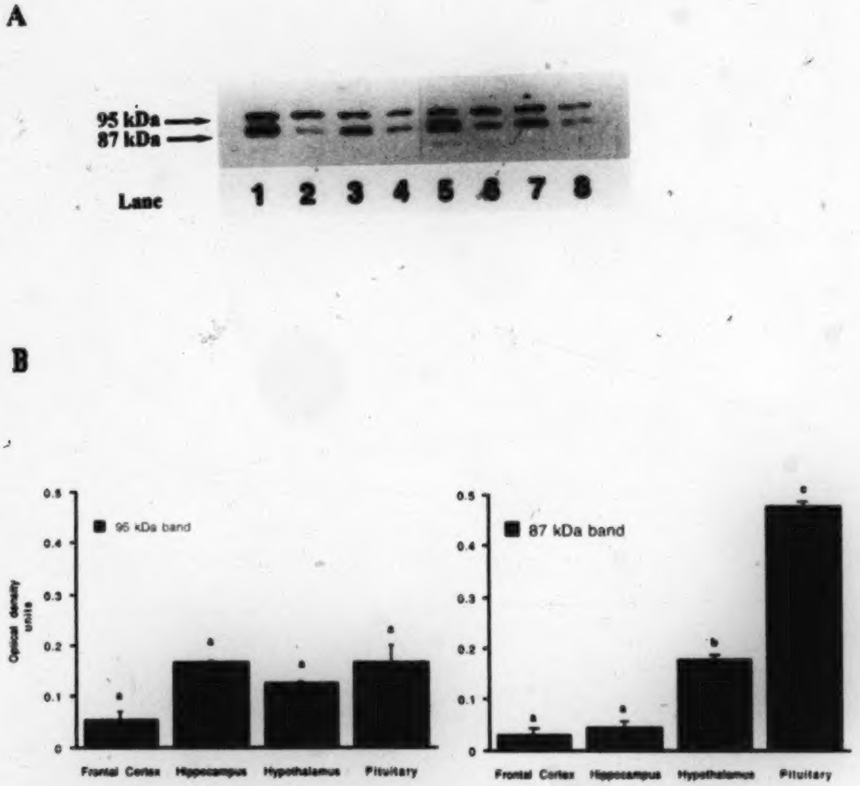

Figure 2-8. Brain region differences in immunoreactive banding patterns. Panel A: western blot for two different animals representative of all 4 animals. Lanes 1 and 5 pituitary gland, lanes 2 and 6 hippocampus, lanes 3 and 7 hypothalamus, lanes 4 and 8 frontal cortex. Proteins (40μg per lane) were separated on a 6% tris-glycine SDS-PAGE gel. Panel B: statistical analysis of relative optical density. Means with different letters are significantly different ($p<0.0001$).

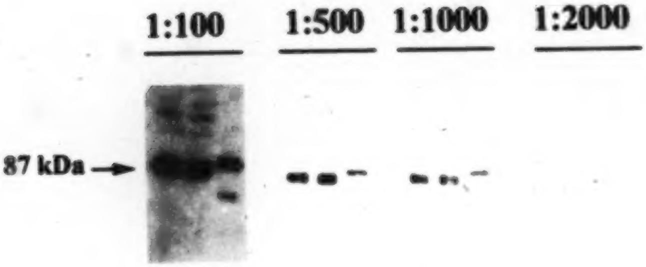

Figure 2-9. Titration of #51 GR antibody in porcine frontal cortex. Four dilutions of #51 GR antibody (1:100, 1:500, 1:1,000, and 1:2,000) were incubated with 2 lanes of frontal cortex tissue from the same pig (first 2 lanes from left to right) and recombinant human GR (third lane).

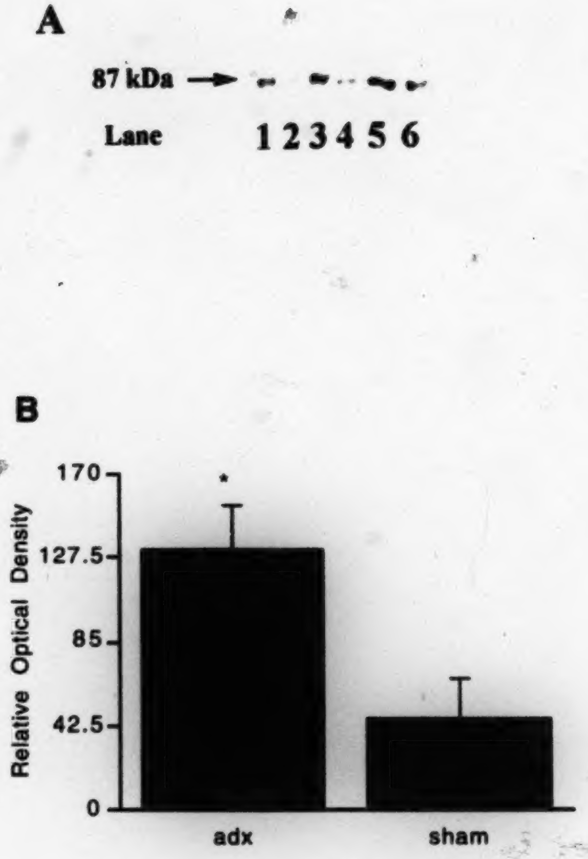

Figure 2-10. Effect of long-term adrenalectomy on hippocampal immunoreactive GR levels in adult male Long-Evans rats. Panel A: representative western blot showing immunoreactive GR levels obtained with the #51 GR antibody with a constant level of protein loaded per lane. Lanes 1,3, and 5 contain hippocampal samples obtained from 3 different adrenalectomized rats with lanes 2, 4, and 6 containing hippocampal tissue from 3 different sham operated rats. B: Graphical representation of group means from relative optical densities. *Significantly different at $p<0.05$.

2.5. DISCUSSION

The series of experiments presented in this chapter describe the initial studies on identifying GR in porcine brain tissue. Initially the #57 antibody from Affinity Bioreagents was used which resulted in the detection of a doublet for GR. Many tissues/cells exhibit a doublet for GR using a variety of GR antibodies (Burnstein et al. 1991, Dallman et al. 1991, Hollenberg et al. 1985, Hutchison et al. 1993, Marks et al. 1986, Sanchez et al. 1994) or a single immunoreactive band (Bellingham and Cidlowski 1989, Cidlowski et al. 1990, Dong et al. 1988). Using tissues obtained from a stillborn pig left in the unit at room temperature, the relative lability of the 87 kDa band was revealed in that it was not detected upon western blotting. In a comparison of species, neonatal female rats exhibited the same hippocampal banding pattern for immunoreactive GR as neonatal pigs, while adult male Long-Evans rats showed a reduced expression of the doublet. This may reflect an age-related difference in hippocampal GR banding pattern between neonatal and adult rats or a sex difference since neonatal rats were female and adult rats used were male. In a comparison of strain, adult Sprague-Dawley appeared to most closely resemble the pattern of doublet expression in the pig and neonatal Long-Evans rat, with banding pattern differences being detected amongst the 4 strains tested here. It was therefore concluded that the #57 GR antibody detected two bands in both rats and pigs and showed different patterns of banding depending on strain, and possibly species, age and gender.

Studies were then carried out to identify which of the two bands represented the functional GR. In previous work in rats we had demonstrated that it was the lower band that responds to ADX and corticosterone replacement in rats and it was concluded that this is the form that is the functional GR in the rat (O'Donnell et al. 1995). The examination of GR covalently labelled with [^{3}H]dexamethasone revealed specific labelling for only the lower 87 kDa band. In addition, the 95 kDa band showed no difference in signal intensity depending on brain site and yet GR expression is known to differ depending on brain region in the rat (Chao et al. 1989). It is believed that the relative intensities of the bands reflects GR concentrations rather than differences in antibody affinity for various forms of GR since the antibody that was used has been shown to recognize all forms of GR (activated, non-activated, and DNA bound) (Burnstein et al. 1991, Cidlowski et al. 1990). From these data it can be concluded that the lower band is the functional GR in terms of ligand binding in the pig, as was also the case for the rat.

In humans there are two isoforms of GR designated α and ß with the α form electrophoretically migrating at 95 kDa while the ß isoform migrates more rapidly at a lower molecular weight (Hollenberg et al. 1985). The ß GR represents an alternative splicing event of GR mRNA resulting in a shorter fragment with a relative mobility for the translated protein of 90 kDa on SDS

PAGE compared to 94 kDA for the α GR (Oakley et al. 1996). In contrast, a specific signal in this range in porcine frontal cortex was not detected and it was concluded that neither of the bands in the doublet was homologous to the human ß GR isoform. A strong signal around 65 kDa was detected with the ß GR antibody. This band was also detected with one lot of the #57 antibody and occasionally when using other lot numbers of this same antibody. Shapiro (1987) showed artifactual 65 kDa keratin bands were recognized by 5 out of 40 rabbit sera tested, which may explain the unknown protein species detected with the supposedly specific ß and #57 GR antibodies. From the results to date, it appears that the 95 kDa band represents a nonspecific band, and in fact, the #57 antibody also reacts with artifactual bands with the relative mobility of 66 kDa. Cidlowski et al. (1990) produces both antibodies and have reported the #57 as detecting a single immunoreactive band; however, more recently they have shown a doublet present in COS-1 cells transfected with α GR and have proposed that this protein, which cannot be ß GR, may be a degradation product or post-translational modification of the 94 kDa human α GR (Oakley and Cidlowski 1996). These data provide further evidence of the questionable specificity of this antibody.

The #51 GR antibody yielded a specific signal at 87 kDa and no doublet, with undetectable nonspecific binding to other proteins. Using this antibody, significant upregulation of immunoreactive GR in the hippocampus upon long-term ADX was detected which are consistent with data previously reported on immunoreactive GR upregulation in the hippocampus using the #57 GR antibody (O'Donnell et al. 1995) and other experiments using receptor binding assays (McEwen et al. 1974, Meaney and Aitken 1985, Sarrieau et al. 1986, Turner 1986). Even though both antibodies do recognize GR in the pig and the rat, the #51 was consistently reliable and showed no non-specific interactions with other proteins. It was therefore decided that the #51 antibody would be used for the remaining experiments described in this thesis, with the exception of those performed prior to testing of this antibody.

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CHAPTER 3

REGIONAL DISTRIBUTION OF GLUCOCORTICOID RECEPTORS IN NEONATAL AND ADULT PORCINE BRAIN TISSUE: EFFECTS OF DIFFERENTIAL TISSUE PREPARATION

3.1. INTRODUCTION

During the first two weeks of life GR levels in the hippocampus of rats are increasing and levels equivalent to adults appear by day 10 *post partum* (Meaney et al. 1985). Neonatal handling treatments in rats during this time period resulted in a permanent upregulation of GR in the hippocampus (Meaney and Aitken 1985). Developmental changes in GR concentrations have not been examined in porcine brain tissue. In fact, the presence or regional differences in GR expression have not been examined in this species. If neonatal handling were to effect hippocampal GR concentrations in pigs it could be hypothesized that a similar neonatal period of hippocampal GR development could also exist in this species. In this chapter we have tested this hypothesis as well as examined the site specific expression of GR using western blotting for GR in selected brain regions in neonatal and adult pigs.

In order to truly take advantage of the improvements in GR detection provided by western blotting, compared to receptor binding assays, an examination of the effects of tissue preparation techniques on GR detection becomes necessary. Receptor binding assays will only detect GR that is in a conformation that is able to bind ligand (Bresnick et al. 1989), whereas with specific antibodies, western blotting can detect all forms of the receptor (Burnstein et al. 1991, Cidlowski et al. 1990, Srivastava and Thompson 1990) and, therefore, may be a more accurate reflection of protein levels. An additional advantage is that the antibodies used will also detect nuclear GR signals (Burnstein et al. 1991, Cidlowski et al. 1990, Srivastava and Thompson 1990). The most common preparation used (since it has been used extensively with receptor binding assays) is the soluble cytosolic preparation which does not include nuclear GRs.

In the current work a detergent based preparation was also used which would ensure disruption of the cell membrane, and prevent microsome formation, and would provide an optimal preparation for the examination of endoplasmic reticular calcium-binding proteins which were examined in Chapter 8. There were advantages in using the above two different approaches since in the soluble cytosolic method, nuclear GRs remained relatively intact and studies examining nuclear translocation could be performed by examining the difference in immunoreactive GR detected in the cytosolic fraction between treatments.

A third technique of high salt extractions in order to liberate all GR from cells (with the exception of 10% of the nuclear GR fraction which is non-salt extractable (Dong et al. 1988) is the method of choice for analysis of total GR receptors. Unfortunately, the high salt used in such preparations affects electrophoretic mobility, necessitating salt removal with low molecular weight exclusion columns or extreme dilution of the sample prior to electrophoresis. More recently, in 1997, a precast gel product became available from Novex which is resistant to the negative effects of high salt concentrations up to 1 M NaCl (Helixx Technologies, product information). The series of studies presented in this chapter examined regional distribution of GR in porcine brain tissue from adult and neonatal pigs using different tissue preparation techniques.

3.2. METHODS

3.2.1. Animals

All experiments were performed under the approval and guidelines of the Canadian Council on Animal Care. Experiments involving pigs used 3 male 14 day old National Pig Development pigs. Pigs were anaesthetized with 40 mg/kg Ketamine HCl i.m. and decapitated upon confirmation of anaesthesia. The pituitary gland was collected and the frontal cortex, hypothalamus, and hippocampus were dissected out on ice within 3 minutes of decapitation. Tissue samples were snap frozen in liquid nitrogen and stored at -72^0 C until analysis. Adult pigs were 3 post-pubertal National Pig Development male pigs euthanized with exanguination after electrical stunning with 3 amperes of current. Tissue collection was the same as described for the neonatal pigs.

3.2.2. Sample preparation

Tissue samples were first powderized in liquid nitrogen using a pestle and mortar. For soluble cytosolic preparations (SC) tissues were homogenized on ice, using a polytron homogenizer, in 3 volumes of ice cold TEDGM buffer (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% (v/v) glycerol, 10mM molybdate, pH 7.4) containing aprotinin (3.5 µg/ml) and phenylmethylsulfonyl fluoride (PMSF) (1.0 mM). The homogenates were centrifuged at 100,000g for 45 minutes at 4°C in a Beckman Ultracentrifuge. The supernatant, which represents a soluble cytosolic fraction, was collected on ice and frozen at -72^{0}C. This centrifugation and collection procedure was used for all three preparations. For the detergent preparation (DET) tissues were homogenized on ice, using an ultrasonic dismembranator (Braun) in a 1:4 dilution of tissue with ice cold TEDGM buffer with 1% Triton X-100 (v/v) (30mM Tris, 1mM EDTA, 2mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate) containing aprotinin (3.5µg/ml), AEBSF (0.4 mg/ml), leupeptin (1 µg/ml) and pepstatin (1µg/ml). For whole cell extract (WCE) preparations, the powderized tissue samples were homogenized on ice using a Vibra Cell sonicator in cold TEDGM (described above) containing 0.4 M NaCl and protease inhibitors (described above). Protein concentrations were determined using the Bradford assay (Bradford 1976) with DET samples diluted 10 fold thereby reducing the detergent concentration to 0.1% (v/v).

3.2.3. Western blotting

Western blotting for soluble cytosolic fractions was performed on samples containing equivalent amounts of protein that were electrophoretically separated in a Novex minigel apparatus (Helixx Technologies) using discontinuous 6% (w/v) or 8% (w/v) Tris-glycine SDS-PAGE gels. Recombinant human GR (Affinity BioReagents, N.J., U.S.A.) was used as a control for antibody detection. The samples were boiled for 3 minutes in equal volumes of 2X sample buffer (0.13M Tris-HCl, 4% (w/v) SDS, 0.005% (w/v) bromophenol blue, 20% (w/v)glycerol, and 5% (v/v) ß-mercaptoethanol). Proteins were transferred to nitrocellulose membranes using the Novex minigel semi-dry transfer apparatus (Helixx Technologies) with Towbin transfer buffer (Towbin et al. 1979) containing 0.03% (w/v) SDS. Membranes were dried for 30 minutes at room temperature and blocked with 5% (w/v) skim milk powder. The primary GR antibody was the polyclonal (rabbit) anti-human glucocorticoid receptor antibody #51 from Affinity

BioReagents, N.J., U.S.A. A 1: 500 dilution of the primary antibody was incubated with the membranes overnight at 4^0C. The membranes were washed with 4 changes of TBST (20 mM tris base, 140 mM NaCl and 0.01% Tween 20) after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody was incubated with the membrane for 1 hour at room temperature. Both antibodies were diluted with TBST containing 0.5% (w/v) skim milk powder. Hyperfilms (Amersham) were exposed to chemiluminescence via a horseradish peroxidase-linked secondary antibody and the ECL detection system (Enhanced Chemi-Luminescence) (Amersham Life Sciences). DET and WCE samples were examined using the NuPAGE electrophoresis system with some modifications. 50 µg of protein per sample was added to a commercially available (Novex) 4X sample buffer (1.17 M sucrose, 563 mM tris base, 423 mM Tris-HCl, 278 mM SDS, 2.05 mM EDTA, 0.88 mM Serva Blue G250, 0.70 mM Phenol Red) with 10% (w/v) dithiothreitol added just prior to denaturing samples at 70^0C for 10 minutes. Samples were electrophoretically separated along with 1 lane per gel containing Rainbow Molecular Weight Markers (Amersham) in a Novex minigel apparatus (Helixx Technologies). Precast 4-12% Bis-Tris NuPAGE gels and a MOPS SDS electrophoresis buffer (1 M 3-(N-morpholino) propane sulfonic acid, 1 M Tris base, 10% sodium dodecyl sulfate, 20.5 mM EDTA) were used along with 0.25% (v/v) antioxidant which was purchased from Novex and added to the inner chamber electrophoresis buffer. Proteins were transferred to PVDF membranes (BioRad) using the BioRad wet transfer apparatus (BioRad) with a modified version of the recommended NuPAGE transfer buffer (25 mM Tris base, 25 mM bicine, 1 mM EDTA, 0.03% (w/v) sodium dodecyl sulfate, 20% (v/v) methanol, and 0.1% (v/v) antioxidant purchased from Novex and added immediately prior to transfer). Membranes were blocked with 5% (w/v) skim milk powder for 45 minutes and washed. A 1: 500 dilution of the #51 anti-GR primary antibody in TBS-T (20 mM Tris base, 140 mM NaCl and 0.01% (v/v) Tween 20) containing 0.5% (w/v) skim milk powder was incubated with the membrane overnight at 4^0C. The membrane was washed with 3 changes of TBST after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody in TBS-T containing 0.5% (w/v) skim milk powder, was incubated with the membrane for 1 hour at room temperature. Hyperfilms (Amersham) were exposed to chemiluminescence via a horseradish peroxidase-linked secondary antibody and the ECL detection system (Amersham Life Sciences). The intensity of bands were analyzed using relative optical densities determined using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catharines, Ontario). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent loading amongst neural tissue samples. A suitable control protein for comparing non-neural tissue (pituitary gland) with neural tissue was not known and protein-loading was simply used. All western blots were performed in at least duplicate.

3.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the Super Anova Statsview software package from Abacus Concepts. Data presentation is in the form of means with standard errors of the means. Within each age group 1 factor ANOVA was used with brain region as the factor tested against the residual followed by *post hoc* tests. Statistical analyses on age related differences in GR concentrations across tissue preparations was performed using a 2 factor ANOVA with age and preparation as main factors tested against the residual, followed by Student-Newman-Keuls *post hoc* tests.

3.4. RESULTS

Figure 2-8A (Chapter 2 page 88) shows the tissue distribution obtained with the traditional SC tissue preparation in neonatal pigs. The highest signal intensity was found in the pituitary gland followed by the hypothalamus. The frontal cortex and hippocampal GR expression were not significantly different between each other, however, they had a significantly lower ($p<0.0001$) level of expression than the hypothalamus and pituitary gland (Figure 2-8B). Figure 3-1 shows the regional distribution of GR in neonatal pigs using the WCE tissue preparation. The pituitary GR becomes non-detectable using a minimal antibody concentration required to detect GR in other brain sites due to increased protein liberation and differences in protein compositions liberated under high salt extractions between tissues (Figure 3-1A). Immunoreactive GR concentrations are highest in the frontal cortex followed by the hippocampus and then hypothalamus with hypothalamic GR signal intensity being significantly lower ($p<0.05$) than that for the frontal cortex or hippocampus (Figure 3-1B).

Tissue from adults revealed a similar regional distribution to that found in neonates using the WCE (Figure 3-2A), however the frontal cortex, hippocampus, and hypothalamus all differed ($p<0.0001$) in the level of expression (Figure 3-2B). As in neonates, the rank order of signal intensity was frontal cortex, hippocampus, and hypothalamus.

Neonatal and adult tissues were prepared using the DET and WCE procedures and a comparison of the effect of preparation, as well as age-related changes in GR, was made using both of the above samples. Figure 14A shows the western blot of adult and neonatal tissue prepared with both techniques electrophoresed on the same gel. There was no significant effect of age on pituitary GR concentrations regardless of the preparation technique (Figure 3-3B). In the hypothalamus (Figure 3-3B) there was a significant ($p<0.0003$) age*preparation interaction, with adult GR concentrations higher ($p<0.0001$) than concentrations found in neonatal pigs if a DET preparation was used and the converse when WCE samples were used. This pattern was at least partially repeated in the frontal cortex and hippocampus (Figure 3-3A) with age*preparation interactions ($p<0.002$ for the frontal cortex and $p<0.0094$ for the hippocampus). In the frontal cortex no significant effect of age was found for the WCE samples however, adult DET samples gave a greater ($p<0.04$) signal than DET samples from neonatal pigs (Figure 3-3B). The hippocampus showed the same pattern of expression as the frontal cortex with no significant difference due to age in WCE preparations and greater ($p<0.0014$) GR expression in adult DET samples compared to baby DET samples (Figure 3-3B).

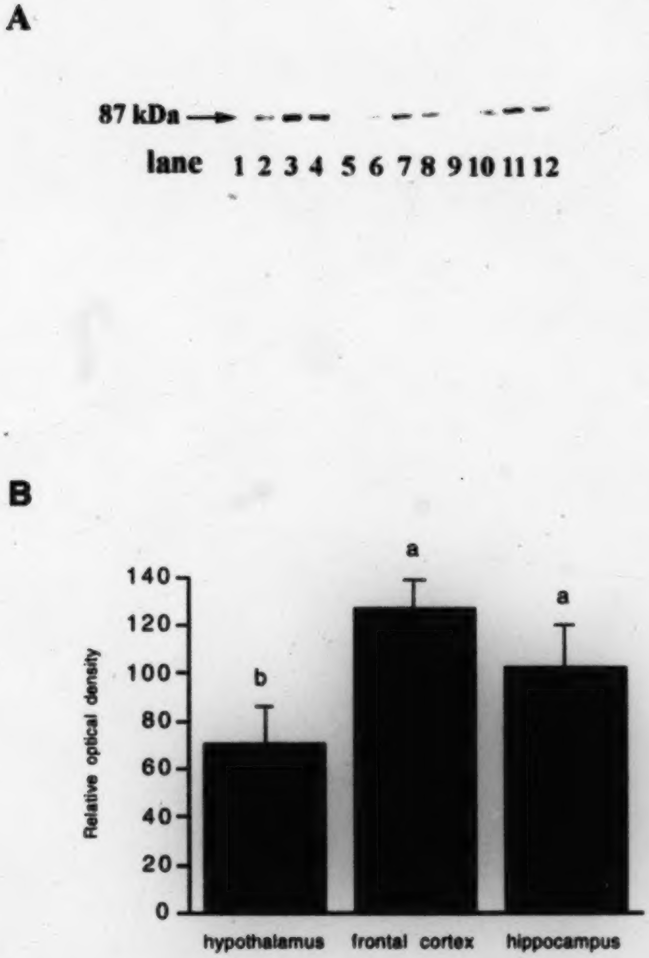

Figure 3-1. Regional distribution of GR in neonatal brain tissue using WCE preparation. Panel A: representative western blot using three pigs. Sample identity is pituitary gland, hypothalamus, frontal cortex, and hippocampus from left to right with no signal for the pituitary gland. Panel B: graphical representation of relative optical densities. Sites with different letters differ significantly ($p<0.05$).

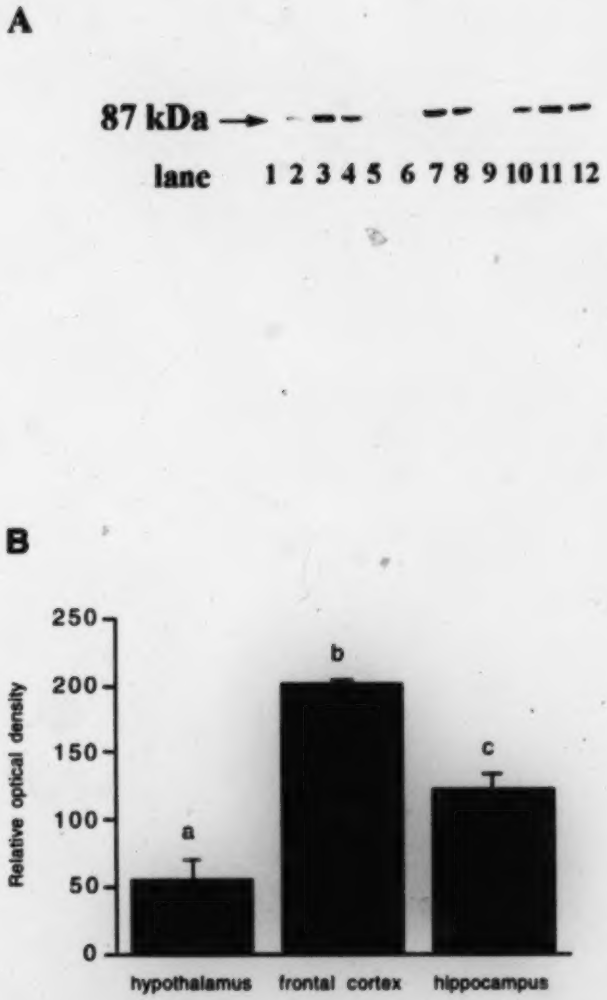

Figure 3-2. Regional distribution of GR in adult brain tissue using WCE preparation. Panel A: representative western blot using three pigs. Sample identity is pituitary gland, hypothalamus, frontal cortex, and hippocampus from left to right with no signal for the pituitary gland. Panel B: graphical representation of relative optical densities. Sites with different letters differ significantly ($p<0.0001$).

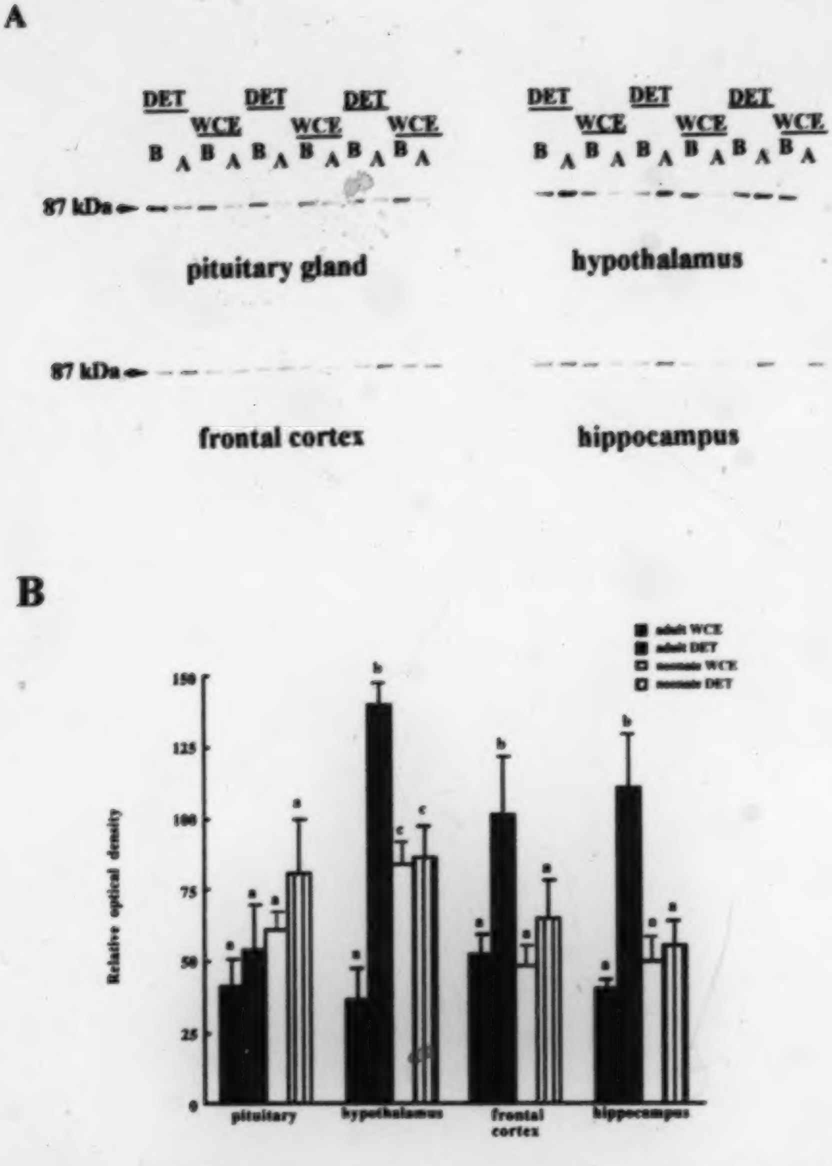

Figure 3-3. Regional distribution of GR in adult and neonatal brain tissue using two different sample preparation techniques. Panel A: representative western blots. Lanes labelled with B are neonatal tissue and A labelled lanes are adult tissue. Pairs of lanes labelled with DET are detergent preparations and pairs labelled with WCE are whole cell extract sample lanes. Panel B: graphical representation of optical densities. Sites with different letters differ significantly ($p<0.0001$ for the hypothalamus, $p<0.04$ for the frontal cortex, $p<0.01$ for the hippocampus).

3.5. DISCUSSION

In this series of experiments the objectives were to test the effects of sample preparation on regional distribution of GR in adult and neonatal porcine brain tissue. In addition, to examine whether differences exist in the level of GR between adult and neonatal pigs.

3.5.1. Regional distribution of GR in brain tissue

Using the SC preparation, which is the same extraction method used in GR binding assays, it was found that the pituitary gland contained the highest level of GR followed by the hypothalamus, with lower levels of expression found in the frontal cortex and hippocampus (see Figure 2-8B Chapter 2 page 88). GR binding capacity in ADX rats has been reported to be highest in the pituitary followed by the hippocampus, cortex and then hypothalamus in adults (Chao et al. 1989, Peiffer et al. 1991, Spencer et al. 1990). In ADX dogs, GR binding capacity was highest in the pituitary gland followed by the hypothalamus, frontal cortex and then hippocampus (Reul et al. 1990). The neonatal pig appears to resemble the ADX dog in GR distribution, at least as judged from SC tissue preparations.

Within SC fractions in ADX and corticosterone-replaced rats, western blotting measures a larger amount of GR than receptor binding assays as it can detect non-translocated receptors that are in a form unable to bind ligand (O'Donnell et al. 1995). Even though western blotting detects more forms of GR in SC extracts, the amount of translocated receptors cannot be quantified as they are not included in the soluble fraction. Dong et al. (1988) have shown that treatment of disrupted tissue samples with 0.4 M NaCl will liberate all but 10% of the nuclear GR. This WCE technique cannot be used in receptor binding assays as it prevents binding of GR to labelled ligand by dissociating the heat shock proteins from the GR complex (Scherrer et al. 1990). Until recently (1997) this extraction has also proved problematic in western blotting, since high salt concentrations are known to disturb electrophoretic mobility of proteins. Using Novex NuPAGE precast gels it was possible to use the WCE preparation without the interference of salts during electrophoresis.

The pituitary gland has been shown to be relatively resistant to nuclear translocation of GR in response to elevated corticosterone (Spencer et al. 1993;1990) and in fact the highest concentrations of GR were detected in the pituitary as seen for rats and dogs with the SC preparation. It was not possible to compare pituitary GR concentrations with neural tissue in WCE preparations due to dramatic reductions in the pituitary GR signal. This most likely results from the liberation of large amounts of proteins which effectively dilute the concentration of GR in pituitary samples. The protein levels in all tissues, as measured by Bradford assays, was increased upon high salt extractions. The increase in protein concentrations in the pituitary was the greatest compared to other sites upon WCE extraction. In addition, antibody concentrations used for neural tissue had to be increased compared to those used for western blotting with SC samples, which could be due to a dilution effect of increased protein liberation. The same situation was found with the DET preparation in that antibody concentrations had to be increased presumably due to a diluting effect of increased protein liberation due to the inhibition of microsome formation which normally would have been separated into the pellet during centrifugation as part of the insoluble fraction.

Under basal conditions, 10-15% of hippocampal GRs are translocated and bound by chromatin in rats (Meaney et al. 1988, Spencer et al. 1993;1990). Restraint stress or exogenous corticosterone at stressor induced concentrations resulted in 50-60% of these receptors being translocated and becoming undetectable by receptor binding assays of SC fractions (Meaney et al. 1988, Spencer et al. 1993;1990). This same effect has been observed in the SC fractions of frontal cortex of rats (Meaney and Aitken 1985). This may be causing the relatively low concentrations of porcine hippocampal and cortical GR that have been detected in the current work in SC fractions (Figure 2-8B Chapter 2 page 88) and in fact it might be predicted that an ADX pig would show a profile similar to the rat with relatively high hippocampal and cortical GR.

The examination of regional distribution of GR using the WCE technique in adult and neonatal pigs showed hippocampal GR concentrations to be significantly lower on a weight per weight of protein basis than cortical GR in adult pigs or equivalent to cortical GR concentrations in neonatal pigs (Figures 3-1A and 3-2A). This leads to the conclusion that the regional distribution of brain GR is unique in the pig with highest concentrations of GR found in the frontal cortex, followed by the hippocampus, and lowest concentrations in the hypothalamus. The distribution in SC samples most likely shows relatively lower cortical and hippocampal GR concentrations, compared to the WCE preparation, resulting from endogenous cortisol-induced translocation of GR in these two structures.

In order to accurately quantify GR receptors, the WCE procedure enables analysis of most of the GR in adrenal-intact animals. Potential technical difficulties associated with high salt concentrations in samples can be overcome by using the Nu-PAGE pre-cast gel system and also by increasing antibody concentrations during immunodetection. Since the Nu-PAGE gel system was not available until 1997, the DET extraction procedure was used for some studies in the following chapters.

3.5.2. Developmental effects on brain GR concentrations

Hippocampal GR binding capacity is low on day 3 of life and increases towards adult values during the second week of life in ADX rats (Sarrieau et al. 1988, Meaney et al. 1985). The GR concentrations in the frontal cortex of ADX rats is low during the first week of life and increases to adult concentrations by the second week of life in a similar manner to that found for hippocampal GR (Meaney and Aitken 1985). In fact, GR in most brain regions is lower in the neonate and increases to adult concentrations by the third week of life as measured using receptor binding assays (Meaney et al. 1993) and *in situ* hybridization for mRNA concentrations (van Eekelen et al. 1987). By contrast, the GR level in the pituitary gland is higher in the neonate than in the adult rat (Meaney et al. 1993). In the current work, no significant developmental effect on pituitary GR concentrations in pigs in either the DET or WCE preparation was found. Using the DET preparation, GR concentrations in the hypothalamus, frontal cortex, and hippocampus were significantly higher in the adult pigs compared to the neonatal pigs which paralleled the situation in ADX rats. However, when the WCE preparation was used, the only significant age-related difference detected was a reduction of hypothalamic GR in adult pigs compared to neonatal pigs. A definitive conclusion on developmental regulation of GR is not possible as the changes in GR concentrations in both preparations are occurring amidst developmental changes in other proteins. As a result, western blotting for one protein is occurring against a background of other proteins. This leads to differences in the percentage of total protein that is GR. It was decided that developmental differences in GR expression would be better analyzed by immunocytochemistry in conjunction with *in situ* hybridization, in order to determine if changes in GR expression are occurring with age as these techniques do not depend upon the assumption of constant protein or mRNA content. In fact, they are based on a constant thickness of tissue which is attainable with modern cryostat-sectioning techniques.

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CHAPTER 4

THE EFFECTS OF NEONATAL SEROTONIN SIGNALLING AUGMENTATION ON BRAIN GR CONCENTRATIONS IN PIGS

4.1. INTRODUCTION

The experiments described in this chapter deal with the effects of neonatal quipazine, a serotonergic agonist, administration in pigs on brain GR concentrations. These experiments were carried out to determine if quipazine treatment to neonatal pigs would mimic the effects of handling on hippocampal GR expression as had been found in rats (Meaney et al. 1989). In commercial barns handling of all litters would be extremely labor intensive and if we could intervene at the levels of 5-HT alternate methods could be employed such as sustained release 5-HT agonists injected at the time of iron injections. In cultures of hippocampal neurons from embryonic day 17 rats, quipazine administration increased GR concentrations, although to a lesser extent, than 5-HT administration (Mitchell et al. 1990). It was therefore hypothesized that if neonatal handling altered GR concentrations in pigs it might do so via increased 5-HT signalling, and exogenous quipazine may induce these effects independent of neonatal handling.

Whether quipazine provides significant binding to 5-HT$_2$ receptors (postulated at the time to be involved in generating increased hippocampal

GR) in the pig is unknown. However, if handling does permanently effect GR concentrations in pigs, and does so via the serotonergic system, it may not necessarily be via the same 5-HT receptor subtype. To avoid such potential problems of specificity, the relatively nonspecific 5-HT agonist(with respect to individual 5-HT receptor subtypes) quipazine was used in order to explore the role of serotonin on the development of central GR concentrations in swine.

There is no information on administration of serotonergic agonists in neonatal swine and therefore an additional objective was to determine whether quipazine induced a cortisol release in neonatal pigs. 5-HT is known to activate the HPA axis in rats (Johnson et al. 1992).

A 7 day clearance period after the final quipazine injection was allowed in order that the immediate drug effects involving proposed increases in cortisol release and subsequent nuclear translocation of GR would not be present in the neonatal pigs. This would allow the determination of whether GR concentrations were upregulated in quipazine treated neonatal pigs. At the time these experiments were performed it was not yet possible to use whole cell extracts for the examination of total GR concentrations as the Novex NuPage gel system was not yet available.

In a preliminary trial (5 saline and 5, 3 mg/kg Quipazine injected pigs) we determined that a dose of 3 mg/kg quipazine induced serotonin syndrome (syndrome characterized by uncontrollable head shakes) (Loscher et al. 1990a) in neonatal pigs, which represented a dose significantly lower than that used in rats (Alper 1990, Fuller and Snoddy 1990, Hemrick-Luecke and Fuller 1996). This suggested that either specific 5-HT receptors are more abundant in the pig compared to the rat on *postnatal* day 1, or that central uptake of quipazine is greater in porcine brain tissue. Due to this preliminary observation, the highest dose of Quipazine used was 2 mg/kg which did not induce 5-HT syndrome in neonatal pigs.

As a measure of HPA function and a test of permanent effects of neonatal 5-HT signalling augmentation on stress responses in pigs experiments were carried out to examine whether differential HPA activation occurred in response to Ketamine injections and euthanasia of 14 day old pigs. This chapter, therefore, presents data examining the effects of neonatal quipazine treatment on cortisol release and immunoreactive GR concentrations in neonatal swine, as well as its effects on cortisol responses to a Ketamine injection stressor in neonatal swine.

4.2. METHODS

4.2.1. Animals

All experiments were performed under the approval and guidelines of the Canadian Council on Animal Care. Eight litters of Camborough X Canabrid pigs (Pig Improvement Canada) housed in a single farrowing room were used for this experiment. Litter size was equalized as much as possible and baby pigs were randomly assigned to one of 4 treatments: saline(n=13), 0.5 mg/kg (n=15), 1.0 mg/kg (n=16), or 2.0 mg/kg(n=13) quipazine dimaleate (2-[-1-piperazinyl]quinoline) (Sigma, St.Louis. MS. U.S.A.) within each litter. The day of birth was designated day 0 and birth weights were recorded, and iron injections and cutting of milk teeth was performed. Pigs were numbered dorsally with permanent felt pen. Baby pigs were injected s.c with saline or the appropriate dose of quipazine in an equal volume of saline from days 1 to 7 postnatally. Injections were performed between 9:00 and 9:30 in the farrowing crate. At no time were litters or individuals removed from the farrowing area.

4.2.2. Tail bleeding

Tail bleeding was performed on day 5, one hour after drug injections on all pigs. Baby pigs were removed from the farrowing room and the tails were soaked briefly in warm water and wiped dry. A diagonal cut was made with a scalpel blade and the tail was "milked" to obtain a 500 µl blood sample. The time from removing the pig from the crate and sample collection was less than 2 minutes. Samples were collected in 1.5 ml eppendorf tubes containing 7.5 USP heparin. Samples were centrifuged at 3,000 RPM for 15 minutes and plasma was then stored at -20^0 C until analysis.

4.2.3. Euthanasia

At 14 days of age 1 male and 1 female from each litter, of randomly chosen treatment, were euthanized for tissue collection (n= 2 males and 2 females per treatment). The remaining animals were used for another experiment not presented in this thesis. Baby pigs were removed from the farrowing room and a tail blood sample was obtained and 40 mg/kg Ketamine HCl was injected i.m. After which the pig was returned to the crate and observed until anaesthesia occurred. The pig was then removed to the adjoining laboratory and decapitation was performed and a trunk blood sample was obtained in a heparin coated tube (Becton Dickenson). Crania were removed and the pituitary gland was collected and the frontal cortex, hypothalamus, and hippocampus were dissected out on ice within 3 minutes of decapitation. Tissue samples were snap frozen in liquid nitrogen and stored at -80^0 C until analysis.

4.2.4. Western blotting for GR

Tissue samples were first powderized in liquid nitrogen using a pestle and mortar. A 100 µl aliquot of powderized tissue was homogenized on ice, using a Vibra Cell sonicator in a 1:4 dilution of sample with ice cold TEDGM buffer (30mM Tris, 1mM EDTA, 2mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate) with 1% (v/v) Triton X-100. The buffer also contained aprotinin (3.5μg/ml), AEBSF (0.4 mg/ml), leupeptin (1 μg/ml) and pepstatin (1μg/ml). The homogenates were centrifuged at 100,000g for 45 minutes at 4°C in a Beckman Ultracentrifuge. The supernatant was collected on ice and frozen at -72°C. Protein concentrations were determined using the Bradford assay (Bradford 1976) with samples diluted 10 fold, thereby reducing the detergent concentration to 0.1% (v/v). Samples containing equivalent amounts of protein (50 μg) were electrophoretically separated in a Novex minigel apparatus (Helixx Technologies) using discontinuous 6% (w/v)Tris-glycine SDS-PAGE gels. The samples were boiled for 3 minutes in equal volumes of 2X sample buffer (0.13M Tris-HCl, 4% (w/v) SDS, 0.005% (w/v) bromophenol blue, 20% (w/v)glycerol, and 5% (v/v) ß-mercaptoethanol). Proteins were transferred to PVDF membranes (BioRad) using the BioRad wet tank transfer unit (BioRad) with Towbin transfer buffer (Towbin et al. 1979) containing 0.03% (w/v) SDS. Membranes were blocked with 5% (w/v) skim milk powder. The primary GR antibody was the polyclonal, (rabbit) anti-human, glucocorticoid receptor antibody #51 (Affinity BioReagents, N.J., U.S.A.) which was used at a dilution of 1: 1000 in TBST (20 mM tris base, 140 mM NaCl and 0.01% Tween 20) containing 0.5% (w/v) skim milk powder and incubated with the membrane overnight at 4°C. The membranes were washed with 4 changes of TBST after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody in TBST containing

0.5% (w/v) skim milk powder was incubated with the membrane for 1 hour at room temperature. Hyperfilms (Amersham, Toronto, Ont. CA.) were exposed to chemiluminescence via a horseradish peroxidase linked secondary antibody and the ECL detection system (Enhanced Chemi-Luminescence) (Amersham, Toronto, Ont. CA. Life Sciences). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent loading amongst tissue samples. All western blots were performed in at least duplicate. The intensity of bands were analyzed using relative optical densities determined using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catharines, Ontario).

4.2.5. Cortisol assay

Plasma cortisol concentrations were measured with GammaCoat™ Cortisol ^{125}I RIA Kit (Incstar Co.) using 25 µl of plasma. This kit has previously been validated for use with porcine plasma samples (Cook et al. 1996). The detection limit of the assay was 2 ng/ml and intra- and inter-assay coefficients of variability were 8% and 10%, respectively.

4.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the Super Anova Statsview software package from Abacus Concepts. Data presentation is in the form of means with standard errors of the means. Split-plot analysis was used with sex as the whole plot and drug treatment as the sub-plot and litter as the replication followed by Student-Newman-Keuls *post hoc* tests. Treatment, litter, litter*sex, and treatment*sex were tested against the residual. Sex was tested against the litter*sex interaction. Analysis of covariance was used to examine growth weights in baby pigs with birth weight as the covariate and data presentation is in the form of adjusted means.

4.4. RESULTS

Quipazine induced a significant ($p<0.05$) increase in plasma cortisol on day 5 at the 2 mg/kg dosage compared to saline or 0.5 mg/kg (Figure 4-1A) with no significant effects of litter, sex, or litter*sex and treatment*sex interactions. At the time of sacrifice on day 14 no significant treatment effect was detected in plasma cortisol prior to Ketamine injection or at the time of decapitation, or in the increase in cortisol (Figure 4-2). There were no significant effects of litter, sex, or litter*sex and treatment*sex for any of the stressor testing plasma samples. Quipazine treatment had no significant effect on DET extracted GR concentrations in any brain region examined (Figures 4-3A and B). There were no significant effects of litter, sex, or litter*sex and treatment*sex for any brain region examined. Growth rates of baby pigs were also not significantly affected by drug treatments (Fig 4-4).

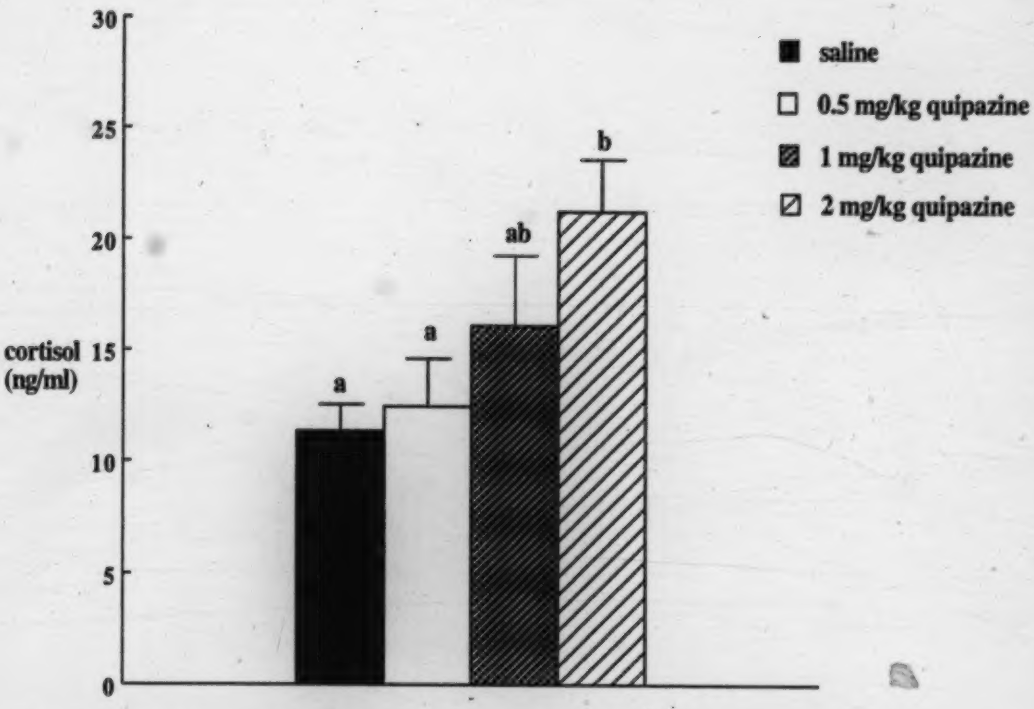

Figure 4-1. Neonatal quipazine administration induced a cortisol release in pigs in a dose-dependent manner. Graphical representation of plasma cortisol concentrations in quipazine (0.5mg/kg n=15, 1mg/kg n=16, 2mg/kg: n=13) and saline (n=13) treated 5 day old pigs. Bars with different letters differ significantly ($p<0.05$).

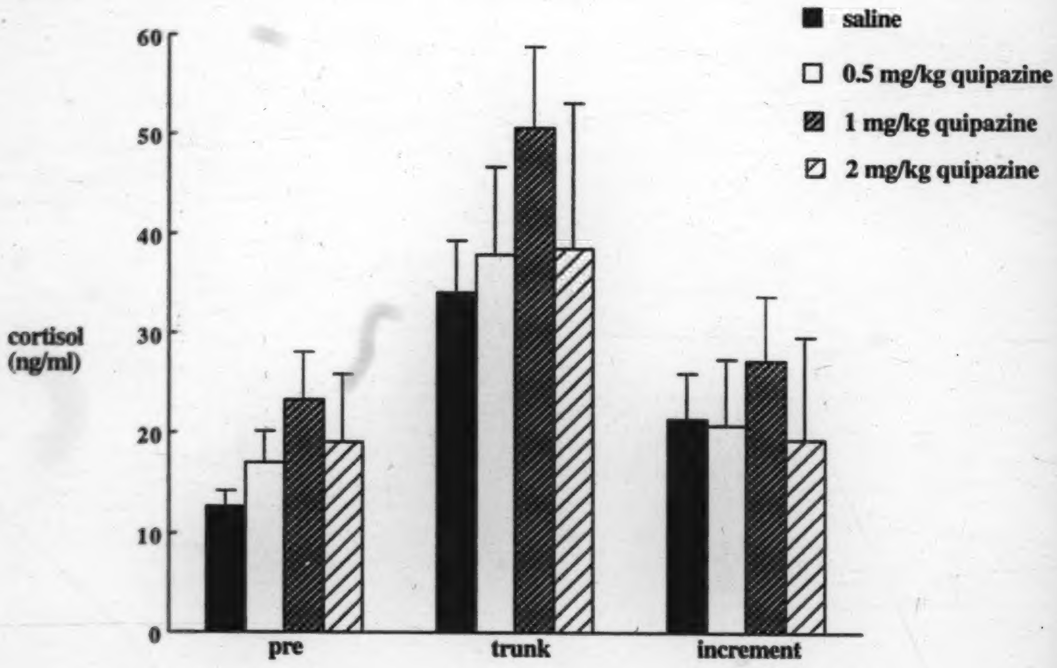

Figure 4-2. Neonatal quipazine treatment did not alter cortisol responses to a Ketamine injection stressor. Plasma cortisol concentrations measured prior to Ketamine anaesthesia (pre), at the time of decapitation (trunk) and absolute increase in cortisol from pre-injection values. (n=4 per treatment).

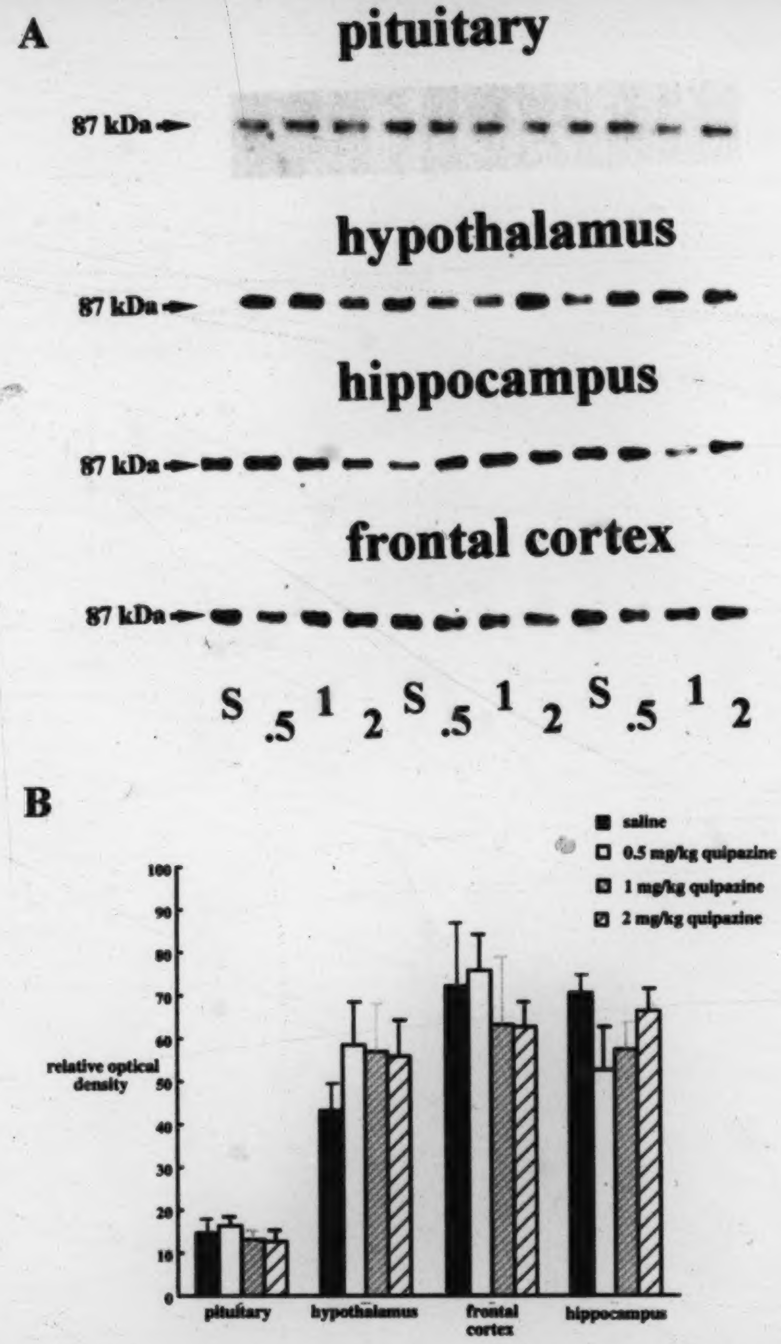

Figure 4-3. Neonatal quipazine treatment did not effect immunoreactive GR levels in selected brain regions. A: representative western blots performed on tissue from 14 day old pigs (S=saline, numerical values represent the dose of quipazine). B: graphical representation of optical densitometry results (n=4 per treatment).

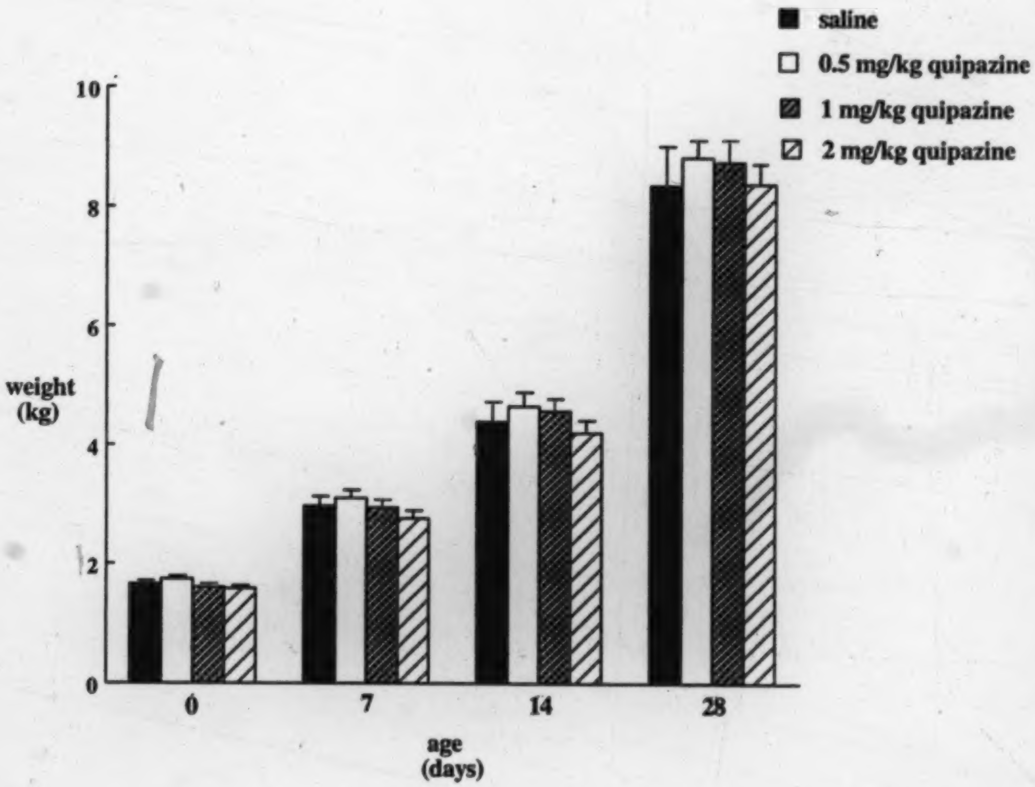

Figure 4-4. Neonatal quipazine treatment did not affect growth rates in neonatal pigs. Growth rates measured weekly on 18 pigs per treatment.

4.5. DISCUSSION

Quipazine administration induced a significant cortisol release in neonatal pigs 1 hour post-injection (Figure 4-1). To our knowledge the effects of quipazine on cortisol release in neonatal pigs has not been examined, therefore, our data represent the first report on the HPA-activating effects of quipazine in neonatal pigs. In rats corticosterone release is elicited by 5-HT via activation of 5-HT$_{2C}$ receptors centrally, via increased CRH release, as well as peripherally at the adrenal cortex (Hemrick-Luecke and Fuller 1996). Any conclusions as to which 5-HT subtypes are involved in eliciting cortisol release in pigs is not possible from the current findings as the agonist used is non-selective: in the rat, it binds 5-HT$_1$, 5-HT$_2$, and 5-HT$_3$ receptors but specifically the 5-HT receptors (Blackshear et al. 1981, Freo et al. 1993). In addition, species differences exist between rats and pigs on the role of 5-HT receptor subtypes in behaviour. Loscher et al. (1990a) reported that 5-HT-induced head shakes are mediated by the 5-HT$_{1A}$ receptor in pigs and the 5-HT$_2$ receptor in rats. Some functional roles may be similar between the two specie for example, the 5-HT$_2$ receptor does mediate hyperthermic and behavioural excitation effects of 5-HT in both pigs and rats (Loscher et al. 1990b). Whether the quipazine-induced cortisol release in neonatal pigs results from activation of 5-HT$_{1A \text{ and } 2C}$ receptors, as in the rat, remains to be determined.

Chronic injections of quipazine did not result in significant differences in body weight gain over the nursing period (Figure 4-4). Neither the augmentation of 5-HT signalling or the increased cortisol induced by quipazine injections affected weight gain in the current experiment. Brown-Borg et al. (1993) have shown that body weights up to 26 days of age did not differ between high and low-cortisol responding pigs to restraint stress. These pigs may be hypothesized to respond to the variety of stressors, present during early life, with greater cortisol responses and therefore may be exposed to greater elevations of cortisol without affecting growth rates. Quipazine administered to 13 day old rat pups inhibited suckling (Bateman et al. 1990) and in adults there is a dose dependent reduction in food intake resulting from a single injection of quipazine (Valencia-Flores et al. 1990). These data suggest that chronic quipazine treatment in pups could conceivably reduce body weights. The effects of chronic quipazine injection in neonatal rats on body weight is not known. The effect of augmented 5-HT on growth rates in neonatal pigs has not been previously reported to our knowledge; however, our results indicate that there is no reduction in growth rate in neonatal pigs chronically administered quipazine.

Plasma cortisol concentrations did not differ significantly between treatments with respect to pre-, post-injection, or in the increment in cortisol (Figure 4-2). The euthanasia was performed 7 days after the last injection of quipazine and therefore it was concluded that there were no permanent effects of neonatal quipazine treatments on cortisol responses to the Ketamine injection. The pigs were tested just 1 week after the final injection however, handling induced increases in GR in rats are present as early as day 7 and it is this change in GR which results in differential corticosterone response to stressors (Meaney and Aitken 1985). It was, therefore, hypothesized that if GR concentrations were altered in neonatally quipazine-treated pigs the effects would be apparent by 14 days after the initiation of treatment and one should see differences in cortisol responses to stressors at this time. To our knowledge the effect of 5-HT signalling augmentation in neonates on HPA responsivity has not been previously examined in pigs.

Neonatal quipazine injections had no significant effect on immunoreactive GR concentrations in any brain region examined (Figures 4-3A and B). Even though the tissue preparation would not have extracted nuclear GRs the lack of treatment effect on cortisol concentrations either before Ketamine injection, or at the time of decapitation, or on the increment in cortisol, indicates that relative nuclear translocation of GR is not likely to differ due to treatment. More recently, the 5-HT_7 receptor has been implicated as the more likely candidate in mediating the handling effects on increasing GR concentrations in the hippocampus in rats (Laplante et al. 1996). The role of 5-HT_7 receptor activation and cent[illegible] GR concentrations in pigs remains to be investigated.

In summary, neonatal quipazine treatments in pigs stimulated the HPA. It did not produce any permanent changes in HPA responsivity with respect to cortisol response to an injection stressor or on central immunoreactive GR concentrations. It can be concluded that neonatal quipazine treatments do not mimic a neonatal handling effect in the pig as has been described in the rat (Meaney and Aitken 1985).

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CHAPTER 5

SEROTONERGIC REGULATION OF BRAIN GR AND HPA RESPONSIVITY IN RATS

5.1. INTRODUCTION

Neonatal handling in rats has been shown to increase GR concentrations in the hippocampus and result in an animal that is relatively hypo-responsive to stressors (Meaney et al. 1992). The mechanism by which GR density was upregulated was via increased turnover of 5-HT in the hippocampus (Mitchell et al 1990). Quipazine, (2-piperazinyl)-quinoline, is a non-selective, non-hallucinogenic, serotonin agonist which binds to 5-HT_1, 5-HT_2, and 5-HT_3 receptors (Blackshear et al. 1981, Freo et al. 1993). Quipazine fully mimicked the effect of 5-HT on GR binding capacity in hippocampal cell cultures albeit at a higher concentration than that used for 5-HT treatments (Mitchell et al. 1990). In the previous chapter, neonatal quipazine administration did not affect changes in GR concentrations in any brain region examined in pigs, which may represent a species difference in serotonergic regulation of GR. In the current study quipazine was used *in vivo* in an attempt to mimic the effects of neonatal handling on HPA function and hippocampal GR concentrations in rats.

High doses of 5-methoxytryptamine (a 5-HT agonist) given to pregnant rats increased 5-HT transporter concentrations, whilst low doses reduced transporter concentrations, in the brain stem and forebrain of adult offspring (Shemer et al. 1991). The authors have reported that this change in 5-HT transporter concentrations is reflective of serotonergic terminal density (Shemer et al. 1991). Since 5-HT is involved in stimulating CRH release from the paraventricular nucleus (PVN) (Chaouloff 1993), the effects of neonatal 5-HT signalling augmentation on 5-HT transporter concentrations in the hippocampus, frontal cortex, and paraventricular nucleus of the hypothalamus were also examined.

Up until the beginning of the third week of life rat pups have been shown to have a blunted ACTH and corticosterone response to a variety of stressors (Kuhn et al. 1990, Walker et al. 1991) compared to adult rats. This period of development has been coined the "stress non-responsive" period (Schapiro et al. 1962). Serotonin signalling augmentation has been shown to induce a corticosterone release using a variety of compounds (Fuller and Snoddy 1990). With a pharmacological increase in 5-HT it was expected that corticosterone release would be increased and therefore nuclear translocation of GR in response to quipazine in neonatal rats was also examined.

In summary, these were three objectives for this series of experiments: firstly, to examine whether augmented corticosterone release, via exogenous quipazine administration, would result in significant GR translocation in the neonatal rats in specific brain structures. Secondly, whether rats treated neonatally with quipazine would differ in HPA responsivity and hippocampal GR mRNA expression as adults was tested. Thirdly, whether neonatal quipazine would produce permanent changes in 5-HT transporter density in the hippocampus, frontal cortex or PVN was examined.

5.2. METHODS

5.2.1. Animals

All experiments were performed under the approval and guidelines of the Canadian Council on Animal Care. Four litters of Long-Evans, hooded rats (Charles River Canada) were randomly assigned to 15 mg/kg quipazine dimaleate (2-[-1-piperazinyl]quinoline) (Research Biochemicals Inc.) or saline injections s.c. from days 1 to 7 of life with birth being designated day 0. Injections were performed in the home cage without separating the mothers and pups. Both male and female pups were treated and female pups (n=11 Quipazine treated and n=10 saline treated) were sacrificed by decapitation 1 hour after injection on day 7 immediately after removal from the home cage and trunk blood was collected in tubes containing 10 µl of saturated EDTA and 100 UIK Trasylol. Blood samples were centrifuged and the plasma was stored at -20^{0}C until assayed for corticosterone. The frontal cortex, hippocampus, and hypothalamus where dissected out and snap frozen in isopentane at -80^0C.

At 90 days of age male rats (n=7 Quipazine treated and n=12 saline treated), were implanted with indwelling jugular catheters under Metophane anesthesia 5 days prior to stressor testing. Animals were restraint-stressed in plastic restrainers for 20 minutes and blood samples were taken 30 minutes prior to stressor application, at 5 and 10 minutes into the stressor application, and again at the termination of the stressor. Recovery samples were taken 20, 60, and 120 minutes post-termination of the stressor. Catheters were non-functional for 5 saline treated rats at the time of stressor testing or basal sampling so blood samples were not obtained from these rats. Two days post-stressor testing basal blood samples were taken for AM and PM ACTH and corticosterone determination at 9:30 and 21:30, respectively. Three days post-basal sampling rats were sacrificed using decapitation and trunk blood was collected and the brain was removed and snap frozen in isopentane at -80^0C and stored at -80^0C until processing for *in situ* hybridization and autoradiography.

5.2.2. Radioimmunoassays

Plasma corticosterone was measured by the radioimmunoassay of Krey et al. (1975) using a highly specific corticosterone antiserum (B3-163;

Endocrine Sciences), [^{3}H] corticosterone (88.6 Ci/mmol; New England Nuclear) and 10 µl of plasma sample. The minimum level of detection with the assay was 10 pg/ml. The antiserum cross-reacts slightly with deoxycorticosterone (4%) but not with cortisol (less than 1%). The intra- and inter-assay coefficients of variability were 8.9% and 11.2%, respectively.

Plasma ACTH was measured by the radioimmunoassay described by Walker et al. (1990) using a specific antiserum at a final dilution of 1:120,000 (IgG Corp.), [^{125}I] ACTH (Incstar), and 25 µl of plasma sample. The ACTH antiserum crossreacts 100% with $\text{ACTH}_{1\text{-}39}$, $\text{ACTH}_{1\text{-}18}$, and $\text{ACTH}_{1\text{-}24}$, but less than 1% with $\text{ACTH}_{1\text{-}16}$, ß-endorphin, α- and ß-MSH and α- and ß-lipotropin. Plasma samples were incubated for 48 hours at 4^0C with antiserum and tracer, then goat anti-rabbit IGg (Peninsula Laboratories) was added and incubated overnight. Bound peptide was obtained by centrifugation at 5000g for 45 minutes. The detection limits of the assay were 1 pg/ml. Intra- and inter-assay variability was 8% and 15% respectively.

5.2.3. Western blotting

In order to determine nuclear translocation of GR the hypothalamus, hippocampus, and frontal cortex samples from 7 day old female pups (5 from each treatment randomly selected) were prepared for western blotting using the soluble cytosolic preparation. Tissues were homogenized on ice, using a Vibra Cell sonicator, in 3 volumes of ice cold TEDGM buffer (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate, pH 7.4) containing aprotinin (1µl/ml) and phenylmethylsulfonyl fluoride (PMSF) (10µl/ml). The homogenates were centrifuged at 100,000g for 45 minutes at 4°C in a Beckman Ultracentrifuge. The supernatant, which represents a soluble cytosolic fraction, was collected on ice and frozen at -72^0 C. Protein concentrations were determined using the Bradford protein assay reagent from Bio-Rad (Bradford 1976). Samples containing equivalent amounts of protein (20 µg) were electrophoretically separated in a Novex minigel apparatus (Helixx Technologies) using discontinuous 6% (w/v) Tris-glycine SDS-PAGE gels. The samples were boiled for 3 minutes in equal volumes of 2X sample buffer (0.13M Tris-HCl, 4% (w/v) SDS, 0.005% (w/v) bromophenol blue, 20% (w/v)glycerol, and 5% (v/v) ß-mercaptoethanol). Proteins were transferred to nitrocellulose membranes using the Novex minigel semi-dry transfer apparatus (Helixx Technologies) with Towbin transfer buffer (Towbin et al. 1979) containing 0.03% (w/v) SDS. Membranes were dried for 30 minutes at room temperature and blocked with 5% (w/v) skim milk powder. The primary GR antibodies used was the polyclonal, (rabbit) anti-human, glucocorticoid receptor antibodies #57 from Affinity BioReagents, N.J., U.S.A. A 1: 4000 dilution of the primary antibody in TBST (20 mM tris base, 140 mM NaCl and 0.01% Tween 20) containing 0.5% (w/v) skim milk powder was incubated with the membrane overnight at 4^0C. The membranes were washed with 4 changes of TBST after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody in TBST containing 0.5% (w/v) skim milk powder was incubated with the membrane for 1 hour at room temperature. Hyperfilms (Amersham, Toronto, Ont. CA.) were exposed to chemiluminescence via a horseradish peroxidase linked secondary antibody and the ECL detection system (Enhanced Chemi-Luminescence) (Amersham, Toronto, Ont. CA.). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent protein loading amongst tissue samples. All western blots were performed in at least duplicate. Intensity of band signals for all results were quantified using relative optical densitometry with the Bio-Rad image analysis system. The relative optical density of the cytosolic GR signal in quipazine treated rats was compared to those treated with saline as a measure of receptor translocation.

5.2.4. *In situ* hybridization

For *in situ* hybridization of GR mRNA, all solutions were prepared in sterile water treated with 0.1% diethylpyrocarbonate. Serial 15 µm thick coronal sections were cut using a Microm HM 500 M cryostat through the dorsal hippocampus (plate 30 corresponding to Bregma -3.14 mm)(Paxinos and Watson 1989) at -15^{0}C and thaw-mounted onto 0.01% (w/v) poly-L-lysine-coated slides. Sections were desiccated overnight at 5^{0}C and then stored at -80^{0}C until use. The GR probe used corresponded to a 674 bp sequence of the rat GR steroid-binding domain subcloned into PGEM plasmid (courtesy of J. Seckle). Plasmids were linearized using *AVA I* restriction enzyme and full length probes were transcribed using T_7 polymerase and 15 µm [^{35}S]-UTP (800 Ci/mmol) (Amersham, Toronto, Ont. CA.). Sense probes were generated using SP6 polymerase and 15 µm [^{35}S]-UTP. Sections were brought to room temperature (10 minutes) and then post-fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 minutes followed by one 5 minute wash in 2X SSC (0.3M sodium chloride, 0.03 M sodium citrate). Sections were then treated with 0.25% (v/v) acetic anhydride added fresh to 1.4% (v/v) triethanolamine and 0.3% (v/v) hydrochloric acid for 10 minutes. Slides were rinsed in 2X SSC followed by progressive dehydration in 50%, 70%, 95%, and 100% (v/v) ethanol prior to delipidation in chloroform for 10 minutes. Sections were then partially rehydrated in 100% followed by 95%(v/v) ethanol. Antisense or sense probes were heat denatured at 65^{0}C and added to hybridization buffer (50% (v/v) deionized formamide, 10 mM dithiothreitol, 10 mM Tris (pH 7.5), 600 mM sodium chloride, 1 mM EDTA, 10% (w/v) dextran sulphate, 1X Denhardt's solution, 100 µg/ml salmon sperm DNA, 100 µg/ml yeast tRNA) to a final concentration of 14 x 10^6

cpm/ml. Hybridization mix (75 µl) was added to each section (1 x10^6 cpm/section) and hybridized for 18 hours at 55^0C in sealed humid chambers. Control sections were hybridized under identical conditions with labelled sense RNA. Following hybridization, slides were rinsed twice in 2X SSC and treated with RNase A (30 µg/ml RNase A, 500 mM sodium chloride, 10 mM Tris, 1 mM EDTA) for 30 minutes at room temperature. Slides were then washed for 5 minutes in 2X SSC at room temperature, 15 minutes at 37^0C in 2X SSC, and for 1 hour at 60^0C in 0.1X SSC. Sections were then progressively dehydrated using 50%, 70%, and 90% (v/v) ethanol in 0.3 M ammonium acetate and air dried and exposed to Bmax film (Amersham, Toronto, Ont. CA.) to ensure the presence of specific labelling. Slides were then dipped in photographic emulsion (Kodak NTB-2) and exposed at 4^0C for 30 days before being developed and counterstained with Cresyl Violet. The hybridization signal within dorsal hippocampal sub-regions was quantified by grain counting over individual hippocampal neurons using a light microscope under brightfield illumination. For each cell field, grains over 40 individual neurons/section were counted and counting was performed on 4 sections per animal. Following subtraction of background, mean values were derived for each hippocampal cell field for each animal. Background ranged between 10 and 15% of values found over hippocampal cells.

5.2.5. Autoradiography

Autoradiography with [^{3}H] paroxetine (5-HT transporter antagonist) was performed using dorsal hippocampal (containing parietal lobe cortical regions) and paraventricular nuclei (plate 25 corresponding to Bregma -1.80 mm) (Paxinos and Watson 1989) 15 μm coronal sections. Sections were brought to room temperature (10 minutes) and then incubated with preincubation buffer (50 mM Tris-hydrochloride, pH 7.7) for 15 minutes at room temperature. Slides were then incubated for 2 hours at room temperature in binding buffer (50 mM Tris-hydrochloride, 120 mM sodium chloride, and 5 mM potassium chloride at pH 7.7) containing either 0.2 nM [^{3}H]paroxetine (19.4 Ci/mmol) (New England Nuclear) for total binding determination or total binding buffer containing 40 μM fluoxetine (5-HT transporter antagonist) for determination of specific binding. Sections were then washed twice in incubation buffer with no ligands for 20 minutes each time followed by dipping in ice cold distilled H_2O and dried under a cold stream of air prior to exposure to ^{3}H Hyperfilm (Amersham, Toronto, Ont. CA.) for 4 weeks at room temperature. Sections were Cresyl Violet stained to ensure the sections contained the region of interest. Average optical densities were determined by selecting the entire region in question and average density within the region was computed using computer-assisted densitometry (MCID Image Analysis System, St. Catharines, Ont.). Standard curves for average density measurements were generated from

Autoradiographic [^{3}H] Micro-scales (Amersham, Toronto, Ont. CA.) which were included in each film cassette. Optical densities were converted to fmol radioligand bound/mg tissue by converting the standard values (nCi/mg) to fmol using the specific activity of the 3H-paroxetine (Ci/mmol). Six sections from each rat were evaluated. Measurements were made on both the right and left sides of the sections and the final values were derived by averaging the twelve sections.

5.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the Super Anova Statsview software package from Abacus Concepts. Data presentation is in the form of means with standard errors of the means. Stress response ACTH and corticosterone data were analyzed using 1 factor ANOVA with repeated measures (time of sample) within each treatment and treatment as the between factor. Pre-stressor values were subtracted from each of the sampling points in order to eliminate any possible effects of altered basal levels. Insufficient litters were used in order to examine possible interactions between treatment and litter and there for the model was a 1 within, 1 between repeated measure. *In situ*, western blotting, and autoradiography group data were analyzed using unpaired t tests.

5.4. RESULTS

5.4.1. Nuclear translocation

Subcutaneous injections of quipazine (15 mg/kg) induced a significant ($p<0.01$) plasma corticosterone response in female rat pups sacrificed on day 7 one hour after quipazine injection (Figure 5-1). This elevated corticosterone induced significant ($p<0.01$ for frontal cortex and hypothalamus, and $p<0.008$ for the hippocampus) nuclear translocation of GR from the cytosolic fraction in all brain regions examined (Figures 5-2A and B). Since the SC tissue preparation will not contain nuclear GRs, what are being detected with the western blots are cytosolic GRs. These are present at a lower level in quipazine treated rats compared to saline injected rats, suggesting a greater nuclear translocation of receptors.

5.4.2. HPA function and GR

There was no significant effect of neonatal quipazine treatment on basal plasma ACTH or corticosterone during the AM nadir or PM peak in adult male rats (Figures 5-3A and B). Stressor induced ACTH concentrations tended to be lower in neonatally quipazine treated rats and significant ($p<0.05$) differences were detected at 20 minutes post-stressor termination (Figure 5-4A). In contrast, corticosterone concentrations did not differ between treatments in response to restraint (Figure 5-4B).

At the time of sacrifice (three days post-basal sampling) cortisol concentrations approximated basal concentrations and there were no significant differences between treatments in plasma corticosterone at sacrifice (Figure 5-5). *In situ* hybridization analysis of GR mRNA showed no treatment effect in any region of the hippocampus (CA1, CA2, CA3, and dentate gyrus) (Figures 5-6A and B).

5.4.3. 5-HT transporter

Optical densitometric analysis of autoradiograms of $[^3H]$paroxetine binding revealed no significant difference in $[^3H]$paroxetine binding throughout the hippocampus or in the frontal cortex (Figures 5-7A and B). In the PVN (three anatomical regions analyzed separately: pamp: medial, palm: lateral, pav: ventral) neonatal quipazine treatment resulted in significantly higher $[^3H]$paroxetine binding in the medial ($p<0.01$) and ventral ($p<0.02$) areas of the PVN (Figures 5-7A and B). The $[^3H]$paroxetine labelling was also higher in the lateral area of the PVN in the quipazine-treated rats; however, this did not reach statistical significance ($p<0.08$).

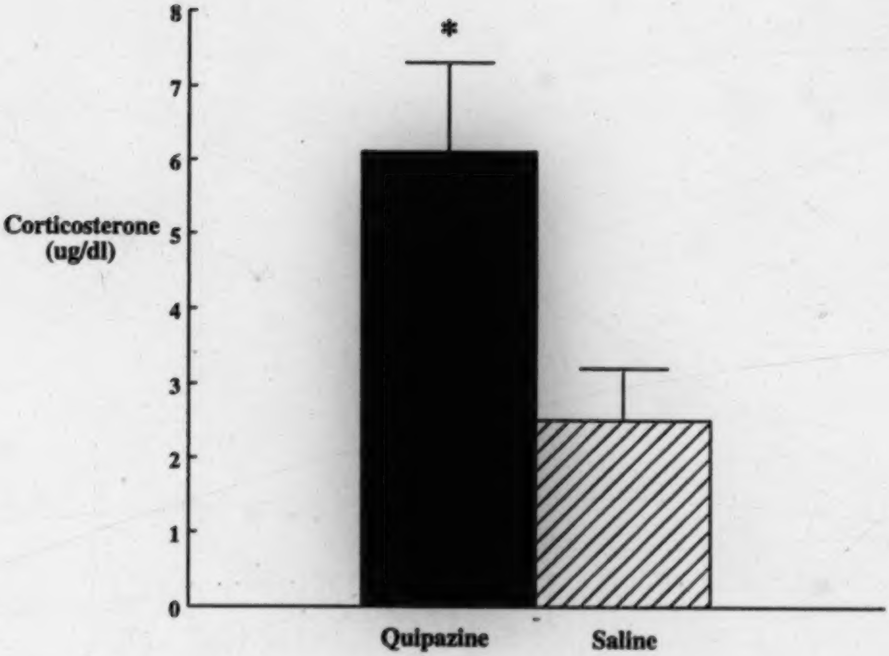

Figure 5-1. Neonatal quipazine administration induced a significant corticosterone release. Graphical representation of plasma corticosterone concentrations in quipazine (n=11) and saline (n=10) treated 7 day old female rat pups. *significantly different at $p<0.01$.

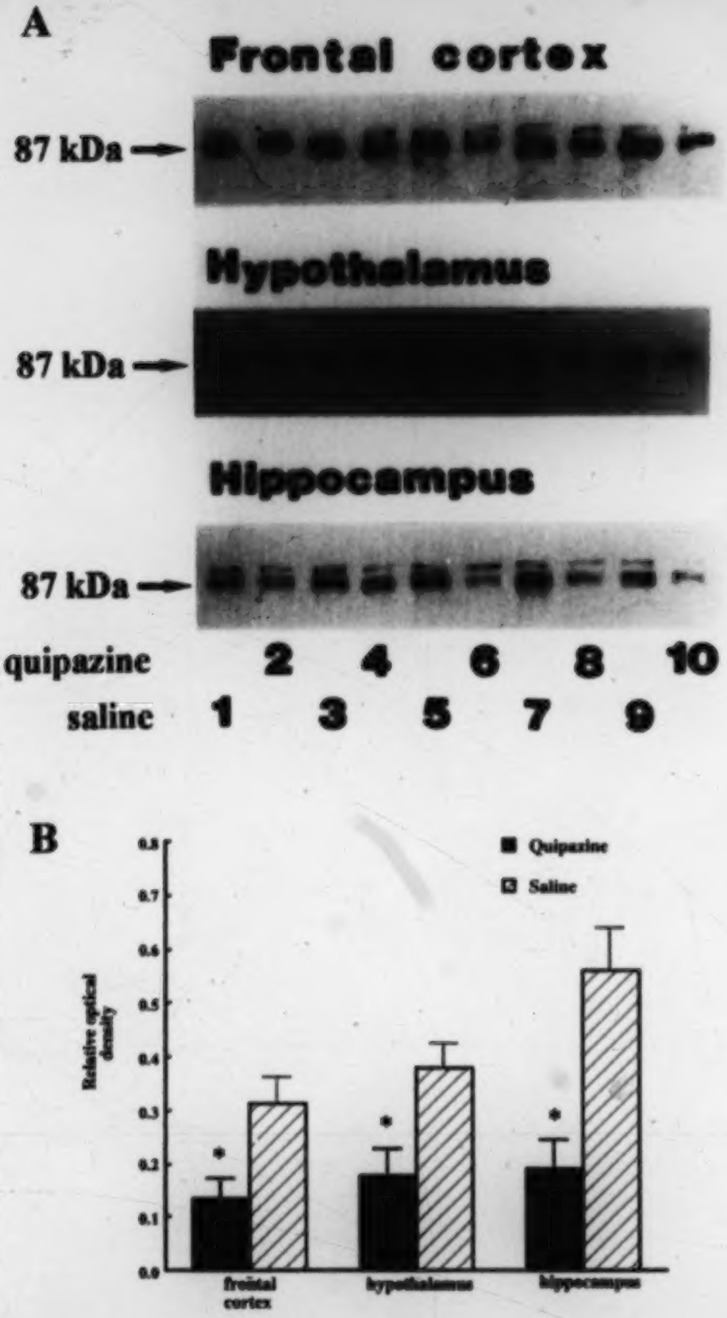

Figure 5-2. Increased plasma corticosterone induced significant GR translocation in all brain regions examined in neonatal female rat pups. A: representative western blots performed on tissue from 10 (n=5 per treatment) 7 day old female pups collected 1 hour post-injection of quipazine. B: graphical representation of optical densitometry results. *significantly different at $p<0.05$.

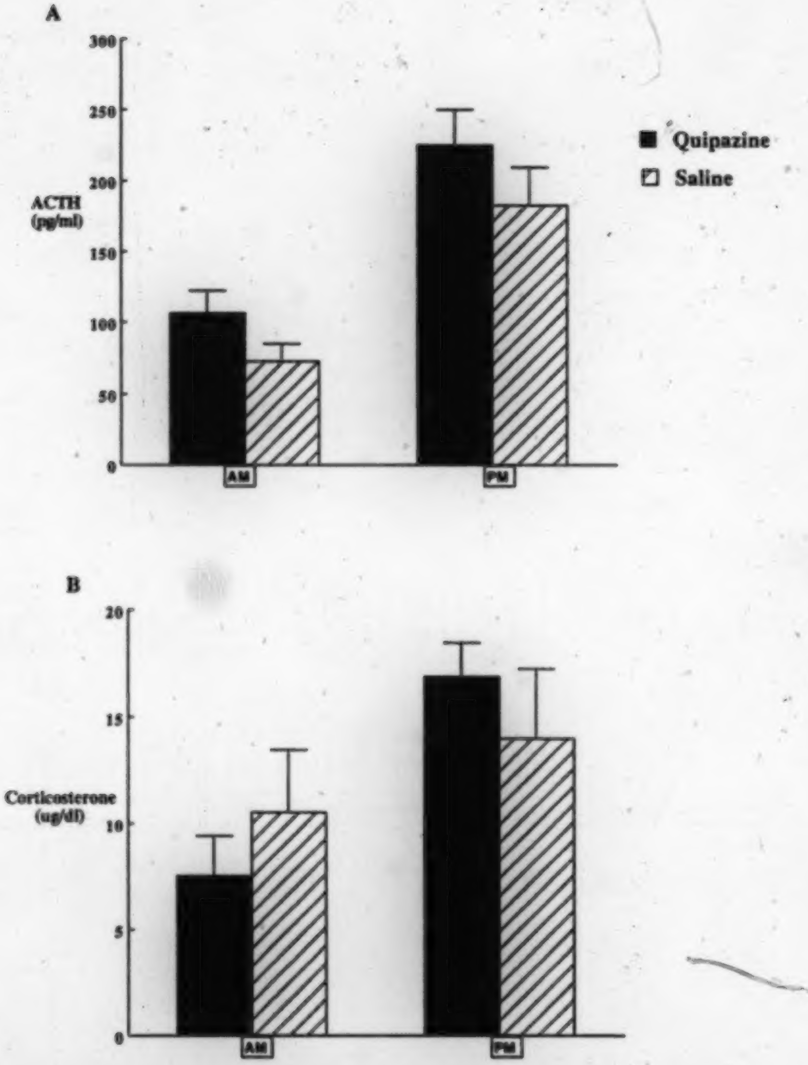

Figure 5-3. Neonatal quipazine treatment did not affect basal plasma corticosterone or ACTH concentrations in adult rats. A: plasma ACTH concentrations measured in the AM (9:30) and PM (21:30) samples (n=7 for each treatment). B: plasma corticosterone concentrations assayed from the same samples.

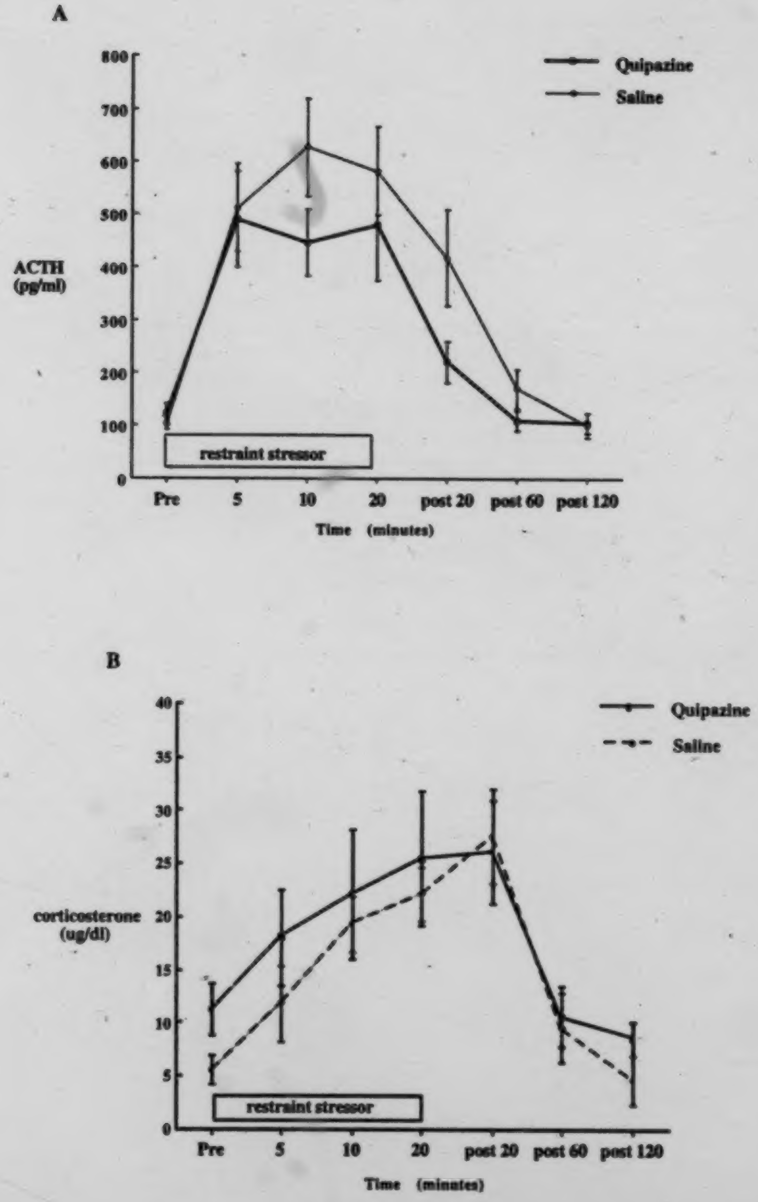

Figure 5-4. Neonatal quipazine treatments resulted in reduced ACTH responses to a restraint stressor with no difference in corticosterone responses. A: plasma ACTH response (n=7 for both treatments). B: plasma corticosterone response. *significantly different at $p<0.05$.

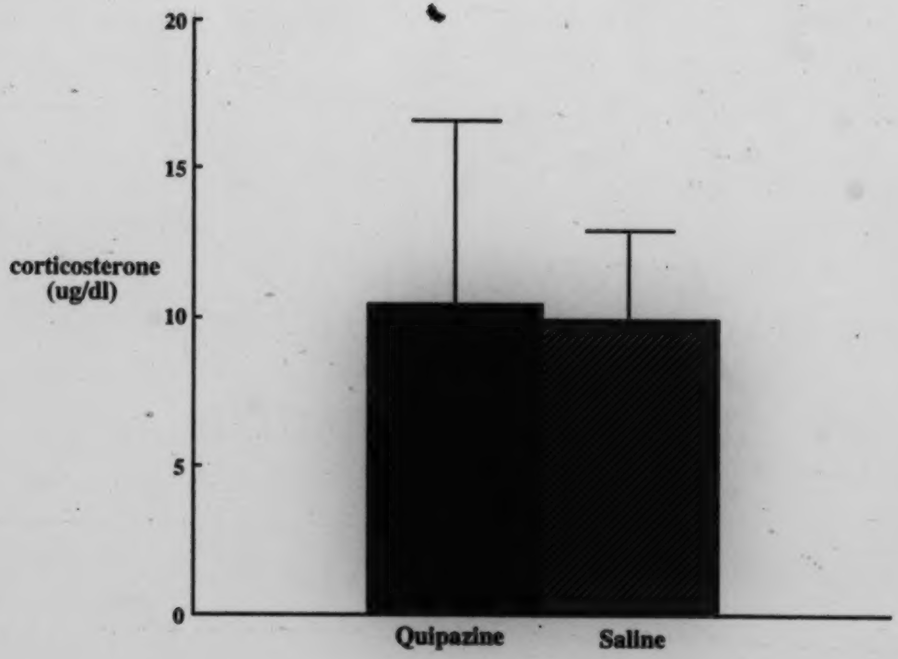

Figure 5-5. Neonatal quipazine treatment had no effect on plasma corticosterone concentrations at the time of sacrifice. Plasma corticosterone concentrations from 7 neonatally quipazine- and 12 saline-treated male rats.

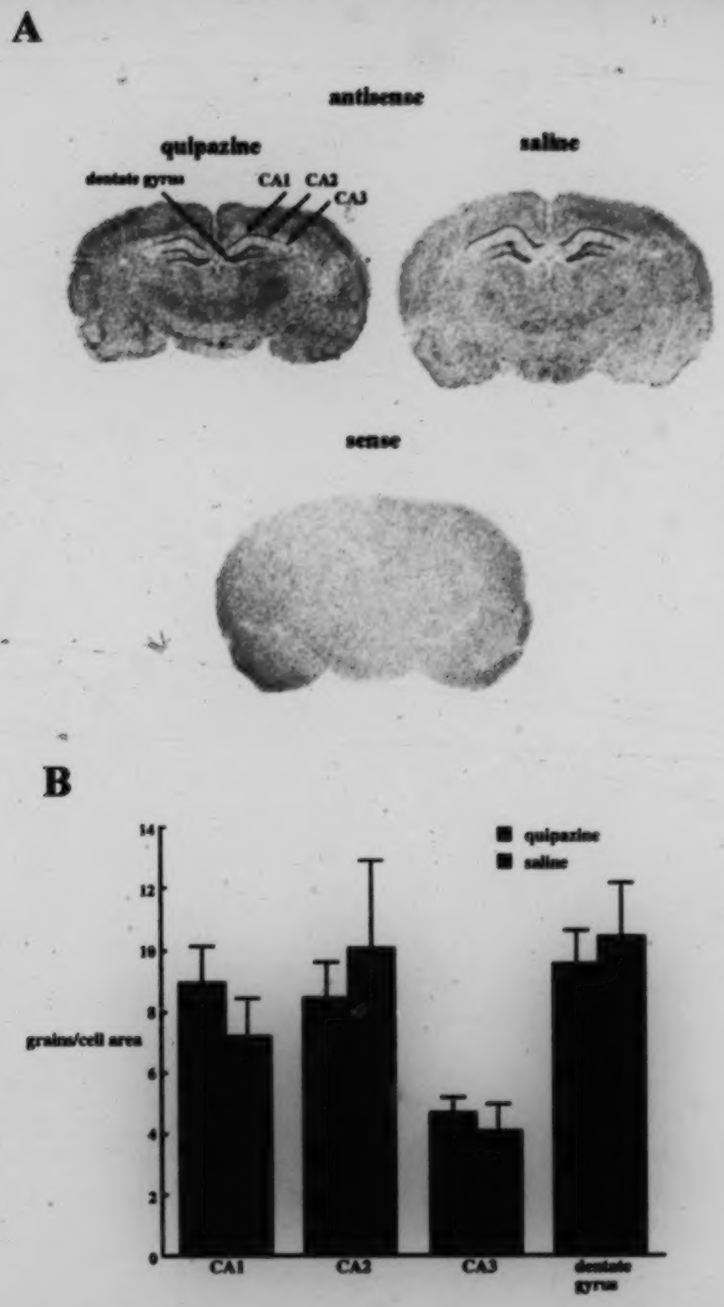

Figure 5-6. Neonatal quipazine treatments did not affect hippocampal GR mRNA levels in adult rats. A: representative autoradiograms from *in situ* hybridization for GR mRNA in a neonatal quipazine-treated adult male rat and a saline treated control along with representative sense autoradiogram. B: graphical representation of grain counting analysis of in situ hybridization results (n=7 for quipazine group and n=12 for saline group).

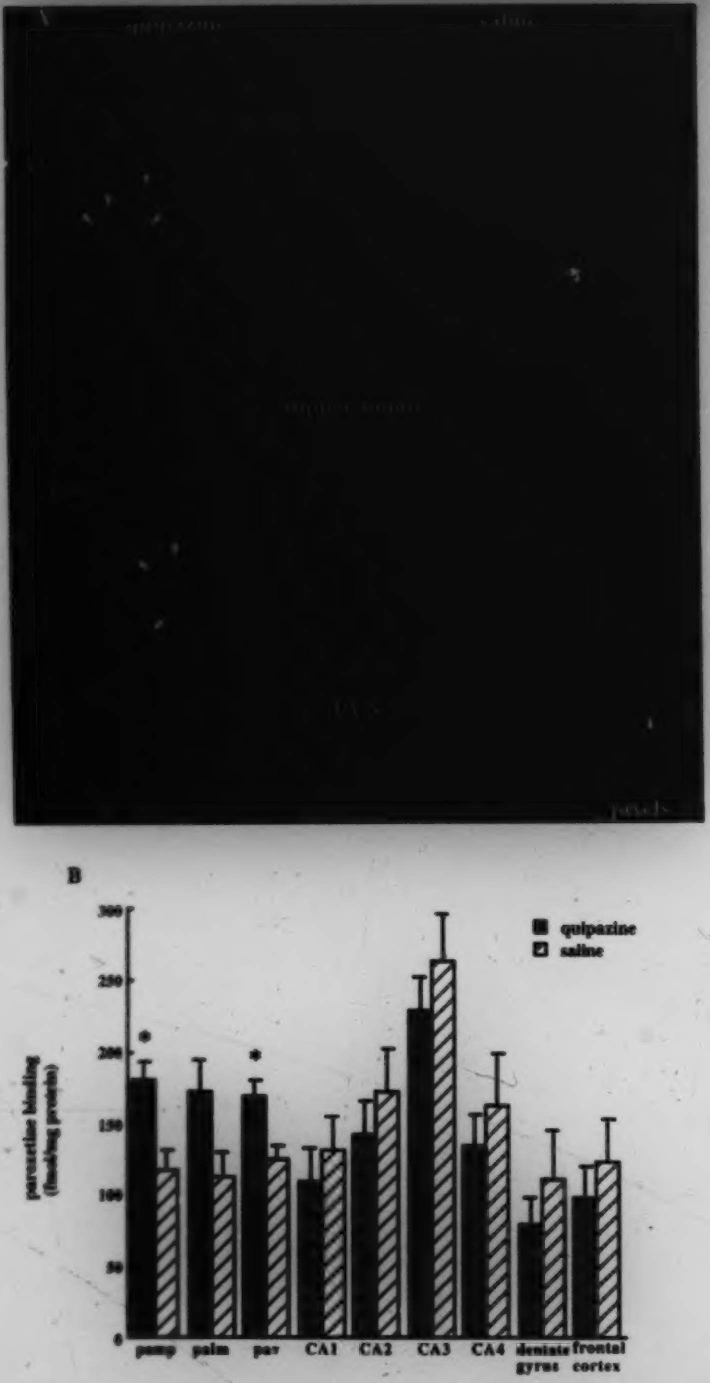

Figure 5-7. Neonatally quipazine-treated rats had significantly higher [^{3}H]paroxetine binding in the PVN. A: representative autoradiograms from neonatally quipazine- and saline-treated adult male rats for the hippocampus, parietal cortex, and PVN. B: graphical representation of average fmol/mg [^{3}H]paroxetine binding in each region examined (n=7 for quipazine group and n=12 for saline group). *significantly different ($p<0.05$). Pamp: medial, palm: lateral, pav: ventral, paraventricular nucleus.

5.5. DISCUSSION

5.5.1. Nuclear translocation

In adult rats a variety of 5-HT agonists, including quipazine, elicit a corticosterone release (Alper 1990, Fuller and Snoddy 1990, Hemrick-Luecke and Fuller 1996). A 2.5 mg/kg s.c. quipazine dose was the lowest dose causing a near maximal corticosterone release of 39 ug/dl one hour after injection in adult rats (Fuller and Snoddy 1990). A significant increase in plasma corticosterone was detected even at the lowest dose of 0.5 mg/kg (20 ug/dl corticosterone) (Fuller and Snoddy 1990). Significant increases in corticosterone also resulted from administration of 8-OH-DPAT, fluoxetine, L-5-hydroxytryptophan and p-chloroamphetamine (Fuller and Snoddy 1990). The effect is via 5-HT$_{1A}$ and $_{2C}$ receptors (Hemrick-Luecke and Fuller 1996) with a partial contribution from peripheral 5-HT$_2$ receptors (Alper 1990). In the present study we found that 15 mg/kg quipazine induced a significant corticosterone release in neonatal rats (Figure 5-1).

On day 2 of life, plasma corticosterone and ACTH, as well as hypothalamic CRF concentrations are low and pups are hyporesponsive to stressors (Levine 1970, Walker et al. 1986). This period, termed the stress-hyporesponsive period (Shapiro et al. 1962) lasts from the second day after birth until the end of the second week of life whereupon basal corticosterone, and circulating ACTH concentrations begin increasing and the pups show a response to stressors equivalent to adult rats (Levine 1970, Walker et al. 1986). In the current work, quipazine elicited a blunted absolute corticosterone response compared to that previously reported in adult rats using much lower doses of quipazine.

Neonatal rats have significantly lower synthesis of CBG by the liver and a reduced half-life of CBG compared to adults (Smith and Hammond 1991, Viau et al. 1996). Viau et al. (1996) have shown that as a result of reductions in CBG, even though the corticosterone response to an ether stressor was significantly lower in 6 day old pups compared to adults, the effective corticosterone signal (translocation of GR to the nucleus) was not different between pups and adults (Viau et al. 1996). Viau et al. (1996) compared concentrations of GR binding with those of ADX pups in order to obtain a percentage translocation value. Meaney et al. (1988) has shown that an immobilization stressor induced approximately 50% of hippocampal GR receptors to translocate to the nucleus in adult rats. In the present experiment it has been shown that quipazine-induced elevations in corticosterone resulted in approximately 50% (or greater in the hippocampus) reduction in soluble cytosolic GR concentrations compared to saline injected controls. This is believed to be reflective of translocation differences in that affects on half life of the receptor are unlikely over such a short period of time (1 hour). This indicated that even at relatively lower elevations in corticosterone, in the neonate, GR translocation equivalent to that found in adults occurred. The largest difference was found in the hippocampus (Figures 5-2A and B) possibly reflecting the greater uptake of corticosterone by this structure (McEwen et al. 1980). The effect of increased corticosterone release in response to quipazine is not expected to confound GR translocation results as exogenous corticosterone given to neonates has no autoregulatory effect on GR concentrations (Meaney et al. 1985).

5.5.2. HPA function and GR

The effect of increasing 5-HT in neonatal rats, on future HPA function, has only been examined using the 5-HT reuptake inhibitor clomipramine administered from days 6 to 18 (Ogawa et al. 1994). These researchers found a significantly lower increase in peak corticosterone concentrations and a faster return to baseline in restraint stressed animals tested at 56 days of age that had been treated neonatally with clomipramine (Ogawa et al. 1994). Possible effects on GR concentrations were not examined in this experiment (Ogawa et al. 1994). In the present study it has been shown that neonatal quipazine resulted in adult rats that had a hypo-stress response, relative to saline injected controls, with respect to ACTH (Figure 5-4A), with no difference in corticosterone response between the groups (Figure 5-4B).

Unlike Ogawa et al. (1994), no significant changes in corticosterone response to restraint stress were seen. However, their study used a 5-HT reuptake inhibitor whereas a 5-HT_1, 5-HT_2, and 5-HT_3 receptor agonist was used in the current study. This agonist targets specific receptors rather than prolonging 5-HT exposure in the synaptic cleft which would conceivably affect all receptor subtypes. More recently the effects of neonatal handling on GR have been postulated to be due to increased activation of 5-HT_7 receptors. Incubation of embryonic hippocampal cells with 50 nM carboxamidotryptamine (5-HT_7 specific agonist) resulted in increased immunoreactive GR as did incubation with 100nM 5-HT (Laplante et al. 1996). The previous work by Mitchell et al. (1990) using quipazine showed that in order to mimic the effect of 5-HT on GR binding capacity *in vitro* a higher concentration of quipazine had to be used relative to 5-HT. In fact, the present study showed no effect of neonatal quipazine treatment on hippocampal GR mRNA (Figure 5-6A and B). The clomipramine used in the Ogawa et al. (1994) study would result in higher 5-HT_7 activation and this may be resulting in the differential results on corticosterone response to restraint which was not seen in the current study.

The differences we have shown in ACTH release in the absence of differences in corticosterone release may indicate that neonatal quipazine treatments are having direct effects on the adrenal gland. In the rat 75% of adrenal medullary cells that contain epinephrine also contain 5-HT

(Holzwarth and Brownfield 1985, Holzwarth et al. 1984, Verhofstad and Jonsson 1983). Alper (1990) has suggested that 5-HT released from the adrenal medulla functions as a paracrine factor therefore providing local control of corticosterone secretion via 5-HT_2 receptors in the adrenal cortex. *In vitro* 5-HT stimulates the synthesis and release of corticosterone from the adrenal cortex (Bing and Schulster 1977). Harbuz et al. (1993) have shown that lesioning 5-HT-containing neurons resulted in increased stress-induced corticosterone concentrations in the plasma with no changes in ACTH response and have hypothesised that 5-HT may have an inhibitory effect at the adrenal gland. It can only be speculated that the quipazine treatments may alter the development of some aspect of the serotonergic system in the adrenal gland which resulted in an elevated corticosterone response to the relatively lower concentrations of ACTH released upon restraint. In fetal sheep cortisol has been shown to enhance adrenal maturation and ACTH responsiveness (Darbeida et al. 1987). An alternate hypothesis, therefore, is that the elevated corticosterone, in response to quipazine administration, is affecting development of the adrenal gland and resultant sensitivity to ACTH.

5.5.3. 5-HT transporter

The trend towards reduced ACTH response at peak stress response and during recovery, with no difference in hippocampal GR mRNA, suggests a possible difference in drive on ACTH release possibly via CRH. Serotonergic innervation of the PVN reveals synapses on CRH-containing neurons in this structure and 5-HT agonists have been shown to induce CRH release via 5-HT_2 receptors (Chaouloff 1993). Serotonin terminals and uptake sites also exist in the pituitary and in fact a locally applied 5-HT_{1A} agonist in rats with transected pituitary stalks elicits ACTH release (Chaouloff 1993). Shimizu et al. (1992) using *in vivo* microdialysis, have shown that an immobilization stressor caused a significant increase in 5-HT release in the hypothalamus. The current findings suggest that neonatal quipazine may have resulted in changes in 5-HT signalling at the PVN.

The 5-HT transporter is found in the presynaptic membrane of terminals and is responsible for terminating the action of 5-HT in the synaptic cleft by removing 5-HT from the cleft and internalizing it back into the neuron (Frazer and Hensler 1994). [^{3}H]paroxetine binds selectively and with high affinity to these 5-HT transporter sites (Arranz and Marcusson 1994, De Souza and Kuyatt 1987, Hrdina et al. 1990). It has been proposed that [^{3}H]paroxetine autoradiography provides a measure of 5-HT nerve terminal density since the uptake sites are found only on terminals (not cell bodies), distribution of [^{3}H]paroxetine corresponds to the distribution of 5-HT nerve terminals, and lesions of 5-HT neurons resulted in decreased [^{3}H]paroxetine binding (De Souza and Kuyatt 1987, Hrdina et al. 1990). Descarries et al. (1995) showed that [^{3}H]citalopram binding in normal, hypo-, and hyper-serotonergic innervated rats was significantly correlated to [^{3}H]5-HT labelled terminals. De Souza and Kuyatt (1987) showed almost identical binding properties of [^{3}H]-paroxetine, and [^{3}H]citalopram therefore the results from Descarries et al. (1995) may be hypothesized to extend to paroxetine.

Lesions of the 5-HT neurons using p-chlorophenylalanine methyl ester decreased 5-HT transporter mRNA concentrations in the midbrain raphe (Linnet et al. 1995). Chronic treatment with antidepressants in adult rats also decreased 5-HT transporter sites in the hippocampus and frontal cortex (Watanabe et al. 1993). It is unlikely that the antidepressants are actually neurotoxic to 5-HT-containing neurons or inhibitory to synapse formation and therefore the authors have suggested that this data reflects a reduction in transporter numbers rather than innervation (Watanabe et al. 1993).

The results of the present study revealed that 5-HT transporters were significantly ($p<0.05$) increased in the PVN in neonatally quipazine-treated rats (Figures 5-7A and B). If these results are interpreted as increased transporter sites per neuron, rather than increased innervation, a reduction in 5-HT content in synaptic clefts in the PVN upon stressor-induced release of 5-HT could occur resulting in a reduced drive on CRH release. Rats treated neonatally with the antidepressant clomipramine (5-HT and norepinephrine reuptake inhibitor) had reduced hypothalamic 5-HT concentrations as adults with no difference in the medulla-pons, frontal cortex, or amygdala/piriform cortex (Feenstra et al. 1996).

There was no significant difference in 5-HT transporter concentrations in the hippocampus or frontal cortex in the present study therefore the increased 5-HT transporter concentrations in the PVN represent structure-specific effects of neonatal quipazine treatment (Figures 5-7A and B). Hilakivi et al. (1987) also showed decreased 5-HT as well as 5-HIAA in the hypothalamus of adult rats treated neonatally with the antidepressants, desipramine and zimeldine. Feenstra et al. (1996) have postulated that this phenomenon may reflect decreased serotonergic innervation of the hypothalamus. Conversely, the results described in this chapter suggests that [^{3}H]paroxetine binding in fact increased rather than decreased and that innervation does not change rather the number of transporters per neuron increased. This could result in increased reuptake of 5-HT and resultant reductions in hypothalamic 5-HT content.

5.5.4. Summary

In this chapter we have demonstrated that even in the presence of low concentrations of corticosterone release (relative to adult rats) significant nuclear translocation of central GR occurs in neonatal rats. The level of translocation is equivalent to that found in adult rats, who release greater amounts of total corticosterone to the same stressor, and therefore the effective GR signal is not hypo-responsive in neonatal rats. Exogenous administration of quipazine during the first 7 days of life does not mimic the neonatal handling effect in rats on hippocampal GRs or HPA responsivity to restraint. It did produce a permanent upregulation in hypothalamic 5-HT transporter concentrations which may have caused the relative hypo-responsivity of ACTH release upon restraint stress due to decreased serotonergic drive on CRH release and decreases in CRH release could then result in decreased ACTH release.

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CHAPTER 6

THE EFFECTS OF NEONATAL HANDLING ON HPA FUNCTION IN PIGS

6.1. INTRODUCTION

In spite of the absence of effects of quipazine on hippocampal GR abundance in pigs it was important to check the effects of neonatal handling itself on HPA function in pigs. It has been shown that neonatal quipazine did not fully mimic the effects of neonatal handling in rats (Chapter 5) and therefore was not a good test of whether the effects of handling occurs across species. More recently, the demonstration of 5-HT$_7$ receptor subtype involvement in upregulating hippocampal GRs (Laplante et al. 1996) suggests that the effects of quipazine may not be identical to handling since 5-HT$_{1,2,and3}$ subtypes are activated with quipazine (Blackshear et al. 1981, Freo et al. 1993) and it is unknown whether any activation of 5-HT$_7$ subtypes occurs with this compound.

The research by Hemsworth's group (Hemsworth and Barnett 1992, Hemsworth et al. 1986a;1986b) indicated that a sensitive period in development exists in pigs during the first 3 weeks of life whereupon environmental manipulations affect permanent changes in behaviour. It is not possible to determine whether these changes could result from a sensitive period in development of hippocampal GR based on data presented in

Chapter 3. As a result, it was decided to examine whether neonatal handling of pigs during the first 2 weeks of life would result in HPA hyporesponsivity and increased brain GRs like that found in rats.

Two possibilities exist for improving the protocols for neonatal handling of swine from that used previously (Hemsworth et al. 1986a). Firstly, the use of the handling procedures used in rats should be applied since this has been shown to result in the permanent attenuation of the stress response. This procedure involved removing the dam and the offspring from the home cage for 5-15 minutes daily for the first 7-21 days of life (Meaney and Aitken 1985). Secondly, appropriate stressors should be used in order to avoid the post-treatment interactions with humans which potentially confounded responses to humans as stressors in the procedures used by Hemsworth (Hemsworth and Barnett 1992, Hemsworth et al. 1986a;1986b). Measures of HPA function in pigs are required in order to ascertain whether handling has the same effects in pigs as it does in rats. This has not been attempted previously in pigs. The significant differences that were detected in the ambulation of artificially reared pigs in open field testing in the initial experiment by Hemsworth's group (Hemsworth et al. 1986b) demonstrated the applicability of this behavioural test for the effects of neonatal handling in pigs. In addition, we examined central immunoreactive GR expression in order to ascertain whether handling upregulated the expression of these receptors as has been found in rats (Meaney and Aitken

1985). The objectives of this experiment were to examine whether neonatal handling altered GR expression, HPA function, or behaviour in pigs.

6.2. METHODS

6.2.1. Animals

All experminets were performed under the approval and guidelines of the Canadian Council on Animal Care. Twelve litters of NPD pigs (National Pig Development), obtained at the Lacombe Research Centre, were used with 6 litters being randomly allocated to the neonatal handling procedure and 6 litters designated nonhandled controls. Litter size was equalized as much as possible using cross-fostering and all litters received iron injections and had milk teeth cut on the day of birth. Tails were not docked and males were not castrated. Birth weight measurement and ear notching for identification were performed on the day of birth. On postnatal day 1 (day of birth defined as postnatal day 0) pigs were removed from the farrowing crate and placed in a straw lined box. The sow was removed from the farrowing crate and placed in a an exercise pen. The duration of separation was for 10 minutes (used previously in the rat handling paradigm) between 9:00 and 10:00 AM for the first 14 days of life. A subset of 12 animals (6 male pigs from each treatment) were weighed and euthanized on day 14 for tissue collection. The remaining animals were weaned at 28 days and housed in groups composed of two litters from the same treatment. At 56 days of age, pigs were moved into the grower/finisher area of the facility and housed in gender-specific groups of 4 with straw bedding until post-pubertal testing of males at 7 months of age (n=11 boars per treatment). Testing of males was performed at the same age which entailed animals being tested on different days. Treatments groups were tested on different days but were interspersed with each other. Animals were fed commercial swine diets (Quality Feeds, Lacombe, Alberta) and water and food were available *ad libitum* throughout the entire experiment. After weaning animals were weighed once per month throughout the experiment.

6.2.1.1. Neonatal procedures

6.2.1.1.A. Euthanasia of 14 day old pigs

Thirty minutes after the termination of handling, at 14 days of age, a subset of 12 male pigs (1 pigs from each litter) were injected with 40 mg/kg Ketamine HCl (Ayerst) i.m. and left in the farrowing crate under supervision until confirmation of anaesthesia. Tail bleeding was performed immediately prior to Ketamine injection (within 2 minutes of removal from the farrowing crate). Pigs were removed from the farrowing area and the tails were soaked briefly in warm water and wiped dry. A diagonal cut was made with a scalpel blade and the tail was "milked" to obtain a 500 µl blood sample. Samples were collected in 1.5 ml eppendorf tubes containing 7.5 USP heparin and centrifuged at 3,000 RPM for 15 minutes and plasma was then stored at -20^0 C until analysis. Anaesthesia induced by Ketamine was confirmed by the lack of pedal reflexes upon which the animals were decapitated. Trunk blood was collected in heparin coated tubes (Becton Dickinson) for plasma cortisol determination. Crania were removed and the pituitary gland was collected and the frontal cortex, hypothalamus, and hippocampus were dissected out on ice within 3 minutes of decapitation. Tissue samples were snap frozen in liquid nitrogen and stored at -72^0 C until analysis.

6.2.1.1.B. Western blotting for GR in 14 day old pigs

Tissue samples were first powderized in liquid nitrogen using a pestle and mortar. A 100 µl aliquot of powderized tissue was homogenized on ice, using a Vibra Cell sonicator in a 1:4 dilution of sample with ice cold TEDGM buffer (30mM Tris, 1mM EDTA, 2mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate) with 1% (v/v) Triton X-100 for DET extraction of GR. The buffer also contained aprotinin (3.5µg/ml), AEBSF (0.4 mg/ml), leupeptin (1 µg/ml) and pepstatin (1µg/ml). The homogenates were centrifuged at 100,000g for 45 minutes at 4°C in a Beckman Ultracentrifuge. The supernatant was collected on ice and frozen at -72^{0}C. Protein concentrations were determined using the Bradford assay (Bradford 1976) with samples diluted 10 fold thereby reducing the detergent concentration to 0.1% (v/v). Samples containing equivalent amounts of protein (50 µg) were electrophoretically separated in a Novex minigel apparatus (Helixx Technologies) using discontinuous 6% (w/v)Tris-glycine SDS-PAGE gels. The samples were boiled for 3 minutes in equal volumes of 2X sample buffer (0.13M Tris-HCl, 4% (w/v) SDS, 0.005% (w/v) bromophenol blue, 20% (w/v)glycerol, and 5% (v/v) ß-mercaptoethanol). Proteins were transferred to PVDF membranes (BioRad) using the BioRad wet tank transfer unit (BioRad) with Towbin transfer buffer (Towbin et al. 1979) containing 0.03% (w/v) SDS. Membranes were blocked with 5% (w/v) skim milk powder. The primary GR antibody was the polyclonal (rabbit) anti-human glucocorticoid receptor antibody #51 (Affinity BioReagents, N.J., U.S.A.) which was used at a dilution of 1: 2000 in TBST (20 mM Tris base, 140 mM NaCl and 0.01% Tween 20) containing 0.5% (w/v) skim milk powder and incubated with the membrane overnight at 4^0C. The membranes were washed with 4 changes of TBST after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody in TBST containing 0.5% (w/v) skim milk powder was incubated with the membrane for 1 hour at room temperature. Hyperfilms (Amersham, Toronto, Ont. CA.) were exposed to chemiluminescence via a horseradish peroxidase linked secondary antibody and the ECL detection system (Enhanced Chemi-Luminescence) (Amersham, Toronto, Ont. CA.). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent protein loading amongst tissue samples. All western blots were performed in at least duplicate. The intensity of bands were analyzed using relative optical densities determined using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catharines, Ontario).

6.2.1.2. Adult procedures

6.2.1.2.A. Open field test

At 7 months of age boars were behaviourally tested for treatment effects using the open field test between 11:00 and 12:00. The open field test was performed in a 9 m x 4 m concrete floored area enclosed with plywood. The area was marked off into 72 equal sized squares ($0.5\ m^2$) using black spray paint with the innermost 16 squares scored as "inner". A radio was suspended above the inner area and was used as a noise stressor. The entrance door was continuous with the plywood sheeting and a viewing window was present at one end of the pen to enable scoring of mobility. A 1 minute adjustment period was given once the boar entered the pen and mobility was recorded for 5 minutes after adjustment. The number of inner and outer squares entered by the left foreleg was recorded. The test pen was not washed between pigs, however, excess urine and feces were removed prior to testing of the next pig. Testing was performed on boars just prior to moving them into farrowing crates.

6.2.1.2.B. Catheterization and blood sampling

Boars were housed in farrowing crates for 4 or 5 days prior to catheterization in order to allow the animals to adjust to confinement. The rear of the line of crates was covered with black plastic in order to provide a visible barrier between the back alleyway where the individual sampling would be located. This was an effective barrier in that if the sampler lifted the black plastic the pigs were noticeably startled. Indwelling ear vein catheters (Cidex sterilized (Surgikos Canada) PE 90 0.8mm i.d., 1.2 mm o.d., Intramedic tubing, Becton Dickinson) were implanted 20-30 cm, under aseptic conditions, in snared boars 4 days prior to sampling. Catheter patency was maintained by leaving the lines filled with heparinized saline. Catheters were not flushed between sampling periods. Extension lines (220 cm of PE 90 tubing with a 16 gauge needle blunted on both ends as a connector) were added to the catheter lines 1 hour prior to sampling and exteriorized through a small hole in the rear blind and heparinized saline was removed and the catheters were flushed with saline. For each sample 2 mls of blood were collected on ice in EDTA-coated 6 ml polypropylene tubes containing 50 µl saturated EDTA and 500 UIK Trasylol (in 50 µl). Hypovolemia was prevented by replacing 2 mls of saline after each sample was obtained and the catheter line was left filled with saline. Blood samples were stored on ice in a refrigerator for no longer than 3 hours and then centrifuged at 3,000 RPM at 4^0C for 15 minutes. Plasma was stored at -80^0C until analysis.

6.2.1.2.C. Basal blood sampling

Five days after catherterization basal blood sampling was performed in undisturbed boars. Boars were fed *ad libitum* at 6:00 and catheter extensions were attached. Sampling times were 8:00, 9:00, 10:00, for AM samples and 14:00, 15:00, and 16:00 for PM samples. During this sampling period no one entered the barn and the sampler remained behind the blind.

6.2.1.2.D. Stressor testing

Two days after basal samples were obtained boars were stressor tested. Catheter extensions were attached at 12:00 and a pre-stressor sample was taken at 13:00. Boars to be stressor-tested on a given day were crated adjacent to each other and nose-snared for 5 minutes at the same time one hour after pre-stressor sampling. Blood samples for the onset and end of restraint were obtained and the barn was vacated. Recovery samples were obtained from the extension lines behind the blind. Samples were obtained 20, 60, 90, and 120 minutes after termination of snaring.

6.2.1.2.E. Dexamethasone suppression

Two days after the completion of stressor testing boars were treated with Dexamethasone. Boars were fed *ad libitum* at 6:00 and catheter extensions were attached. Boars were injected with 0.04 mg/kg Dexamethasone (Sebranek et al. 1973) (suppresses ACTH and cortisol release by binding to GR receptors) via the extension line at 8:00 and the drug was delivered by flushing the line with 5 mls of saline. Blood samples were obtained from behind the blind at 9:00, 10:00, and 11:00 with no one entering the barn from 7:00 until testing was completed.

6.2.1.2.F. Euthanasia of adult boars

Four days after completion of dexamethasone-suppression testing, boars were returned to the grower/finisher area and housed singly until euthanasia 5 days later. Boars were transported to an on-site meat facility at 6:00 and all animals were sacrificed by 10:00. Live weights were obtained and the animals were electrically stunned and exsanguinated. Craniotomies were performed using a hand held circular saw and the brains were collected along with the pituitary gland and snap frozen in liquid nitrogen and stored at -80^{0}C. Blood was collected from the stick wound in polypropylene EDTA coated tubes containing 100 µl of saturated EDTA and 1000 UIK Trasylol (100 µl) for plasma cortisol and ACTH measurement.

6.2.1.2.G. Carcass evaluation

Live animal weights were recorded as well as trimmed hot and cold weights (24 hours *post mortem*) of sides. Lean and fat depths were obtained using a Hennessy grading probe, placed between the last 2 ribs, and a predicted percentage lean yield was obtained.

6.2.1.2.H. Plasma corticosteroid-binding globulin assays

Plasma corticosteroid binding capacity (CBC) was measured using a method previously described by Martin et al. (1977). Samples used were those collected on the basal sampling day and therefore 6 samples for each animal collected at 8:00, 9:00, 10:00, 14:00, 15:00, and 16:00 were assayed. Endogenous steroid was removed from the plasma by passing a 20 µl aliquot through a 10 cm x 1 cm Sephadex LH-20 column and the plasma was diluted 50:1 with TEDGM (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate, pH 7.4). Diluted plasma (225 µl) was incubated with 150 µl of TEDGM containing a previously determined (Martin et al. 1977) saturating 80 nM concentration of [^{3}H]cortisol for 90 minutes at 4^{0}C. Non-specific binding was determined in parallel using a 200-fold excess of cold cortisol. Bound cortisol was separated from free cortisol using 10 cm x 1 cm Sephadex LH-20 columns in triplicate for both the specific and non-specific incubations. The bound cortisol was eluted with 500µl of TEDGM into mini-vials into which 5 mls of Ecolite scintillation cocktail was added and the [^{3}H]cortisol was counted with a Beckman scintillation counter at 45% efficiency for 5 minutes per vial. Protein content in the diluted stripped plasma was determined by the method of Bradford (Bradford 1976) using BioRad Bradford dye and results were expressed as picomoles binding/mg of protein. Free cortisol concentrations were calculated from the total molar concentrations of cortisol and corticosteroid binding capacity as previously reported (Martin et al. 1977) according to a modification of the mass equation (Plymate et al. 1987):

$$(x/B-X)(1/CBC)=K[1-(x/CBC)];\ x=(b-\sqrt{b2-4a})/2$$

where x is the molar concentration of free cortisol, B is the total molar cortisol concentration, K is the association constant of cortisol (3.4×10^{8} M^{-1}) (Kattesh et al. 1980) and CBG, a=BxCBC, and b=1/K+CBC+B. Molar CBC used for calculations was the value obtained for each animal during basal sampling for free basal determinations and average AM values for dexamethasone suppressed cortisol and cortisol concentrations at the time of slaughter. To determine free cortisol concentrations during stressor testing an average of the PM basal CBC values were used since stressor testing was performed in the PM.

6.2.1.2.I. Radioimmunoassays

Plasma cortisol concentrations were measured with GammaCoat^{TM} Cortisol ^{125}I RIA Kit (Incstar Co.) using 100 µl of plasma. This kit has previously been validated for use with porcine plasma samples (Cook et al. 1996). The detection limit of the assay was 2 ng/ml and intra- and inter-assay coefficients of variability were 8% and 10%, respectively.

Plasma ACTH was measured by the radioimmunoassay described by Walker et al. (1990) using a specific antiserum at a final dilution of 1:120,000

(IgG Corp.), [^{125}I] ACTH (Incstar), and 200 µl of plasma sample. The ACTH antiserum crossreacts 100% with ACTH$_{1\text{-}39}$, ACTH$_{1\text{-}18}$, and ACTH$_{1\text{-}24}$, but less than 1% with ACTH$_{1\text{-}16}$, ß-endorphin, α- and ß-MSH and α- and ß-lipotropin. Plasma samples were incubated for 48 hours at 4^{0}C with antiserum and tracer, then goat anti-rabbit IGg (Peninsula Laboratories) was added and incubated overnight. Bound peptide was obtained by centrifugation at 5000g for 45 minutes. The detection limits of the assay were 1 pg/ml. Intra- and inter-assay variability was 8% and15%, respectively.

6.2.1.2.J. Whole cell extract western blotting for GR in adult tissue

The hypothalamus, frontal cortex, and hippocampus was dissected from frozen whole brains post-freezing on a randomly chosen subset of boars (n=6 handled and n=7 nonhandled). Brain tissue was allowed to come to approximately -20^0 C and tissue was dissected using ice-cooled tools. Tissue samples were then powderized in liquid nitrogen using a pestle and mortar. For whole cell extract preparations, the 100 µl of powderized tissue samples were homogenized on ice using a Vibra Cell sonicator in 3 volumes of cold TEDGM (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% (v/v) glycerol, 10mM molybdate, pH 7.4) containing 0.4 M NaCl and protease inhibitors (aprotinin (3.5µg/ml), AEBSF (0.4 mg/ml), leupeptin (1 µg/ml) and pepstatin (1µg/ml)). The homogenates were centrifuged at 100,000g for 45 minutes at

4°C in a Beckman Ultracentrifuge. The supernatant was collected on ice and frozen at -80°C. Protein concentrations were determined using the Bradford assay (Bradford 1976). 50 µg of protein per sample was added to a commercially available (Novex) 4X sample buffer (1.17 M sucrose, 563 mM tris base, 423 mM Tris-HCl, 278 mM SDS, 2.05 mM EDTA, 0.88 mM Serva Blue G250, 0.70 mM Phenol Red) with 10% (w/v) dithiothreitol added just prior to denaturing samples at 70°C for 10 minutes. Samples were electrophoretically separated along with 1 lane per gel containing Rainbow Molecular Weight Markers (Amersham, Toronto, Ont. CA.) in a Novex minigel apparatus (Helixx Technologies). Precast 4-12% Bis-Tris NuPAGE gels and a MOPS SDS electrophoresis buffer (1 M 3-(N-morpholino) propane sulfonic acid, 1 M tris base, 10% sodium dodecyl sulfate, 20.5 mM EDTA) were used along with 0.25% (v/v) antioxidant which was purchased from Novex and added to the inner chamber electrophoresis buffer. Proteins were transferred to PVDF membranes using the BioRad wet transfer apparatus (BioRad) with a modified version of the recommended NuPAGE transfer buffer (25 mM Tris base, 25 mM bicine, 1 mM EDTA, 0.03% (w/v) sodium dodecyl sulfate, 20% (v/v) methanol, and 0.1% (v/v) antioxidant purchased from Novex and added immediately prior to transfer). Membranes were blocked with 5% (w/v) skim milk powder for 45 minutes and washed. A 1: 500 dilution of the primary antibody (anti-GR antibody #51 from Affinity BioReagents, N.J., U.S.A.) in TBS-T (20 mM Tris base, 140 mM NaCl and 0.01% (v/v) Tween 20) containing 0.5% (w/v) skim milk powder was incubated with the membrane overnight at 4^0C. The membrane was washed with 3 changes of TBST after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody in TBS-T containing 0.5% (w/v) skim milk powder was incubated with the membrane for 1 hour at room temperature. Hyperfilms (Amersham, Toronto, Ont. CA.) were exposed to chemiluminescence via a horseradish peroxidase linked secondary antibody and the ECL detection system (Amersham, Toronto, Ont. CA.). The intensity of bands were analyzed using relative optical densities determined using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catharines, Ontario). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent protein loading. All western blots were performed in at least duplicate.

6.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the Super Anova Statsview software package from Abacus Concepts. Data presentation is in the form of means with standard errors of the means. Plasma cortisol levels for 14 day old pigs over injection stressor were tested using 1 factor ANOVA with repeated measures (time of sample) within treatment and treatment as the between factor. The increment in cortisol was tested using unpaired Students t test. Insufficient pigs representing litters were present in order to ascertain the effects of litter. Neonatal GR concentrations were analyzed using unpaired Students t test for each brain region. Plasma cortisol, ACTH and CBC data obtained from basal samples, stressor testing, and dexamethasone suppression tests, as well as body weights, were analyzed using 1 factor ANOVA with a repeated measures factor (time of sample) within each treatment and treatment as the between factor followed by *post hoc* tests. Open field test data were analyzed using unpaired Students t test as insufficient pigs representing the litters were available to test the effects of litters. Correlation analysis and simple regressions were used to analyze relationships between plasma cortisol and open field test mobility scores. Carcass data and slaughter weights were analyzed using analysis of covariance and Student Neuman Keuls *post hoc* tests using the age at slaughter as a covariant. Means and standard errors are presented as adjusted values. A 50% catheter failure rate occurred with remote sampling using ear vein catheters, therefore, the number of animals that plasma samples were obtained for during each test is reported at the bottom of the respective graphs. Correlational analysis was performed for locomotor behaviour and post-stressor termination plasma cortisol on animals in which plasma samples were obtained at that particular time point. In addition, insufficient boars representing individual litters were available for analyzing litter*treatment interactions so unfortunately this analysis was not possible.

6.4. RESULTS

6.4.1. Neonates

There was no significant effects of treatment on pre- and post-injection plasma cortisol concentrations, however, there was a significant effect of time ($p<0.003$) and a significant ($p<0.008$) treatment*time interaction (Figure 6-1). To further explore this interaction analysis of the increment in cortisol revealed that handled pigs had significantly ($p<0.07$) lower increases in cortisol concentrations in response to the Ketamine injection procedure. Immunoreactive GR concentrations did not differ significantly, as a function of neonatal handling, in 14 day old pigs in any brain region examined (Figures 6-2A and B).

6.4.2. Adults

6.4.2.A. HPA function

Figures 6-3A, B, C, and D shows the basal plasma hormone and CBC concentrations measured in post-pubertal boars. No significant effects of treatment or time*treatment interactions were detected for basal plasma ACTH concentrations (Figure 6-3A). A significant ($p<.0019$) effect of time was detected indicating that plasma ACTH concentrations decreased over time. The neonatal handling treatment had a significant effect on plasma CBC with handled boars having significantly ($p<0.05$) higher CBC (Figures 6-3B) with no significant effects of time or time*treatment interactions. Treatment also had a significant (p<0.02) effect on basal total and free cortisol concentrations with handled boars having lower basal plasma cortisol concentrations (Figures 6-3C and D). No significant effects of time or treatment*time interactions were detected for total or free basal plasma cortisol levels.

A 5 minute snaring stressor resulted in no effect (p<0.11) of treatment or treatment*time interactions on plasma ACTH concentrations; however, handled boars tended to have higher plasma ACTH concentrations (Figure 6-4A). No significant (p<0.10) effect of treatment was found on total cortisol concentrations during or after a snaring stressor (Figure 6-4B). A significant (p<0.05) effect of treatment was found on free cortisol concentrations and treatment*time interaction (p<.05), which appeared to be due to reduced plasma free cortisol concentrations in handled boars during the late recovery phase (Figure 6-4C).

No significant treatment effects or treatment*time interactions were found on dexamethasone-induced suppression of plasma ACTH (Figure 6-5A), total cortisol (Figure 6-5B), or free cortisol (Figure 6-5C). The lack of difference in feedback was also reflected in the lack of treatment effects on immunoreactive GR concentrations in any brain region examined (Figures 6-6A and B).

6.4.2.B. Behaviour

Neonatal handling resulted in significantly (p<0.05) greater locomotor score over inner squares and a significantly lower ratio of outer:inner squares entered in adult pigs (Figures 6-7A and B). The total number of squares entered was significantly ($p<0.05$) negatively correlated with both total and free plasma cortisol concentrations 90 minutes post-snaring (Figures 6-7C and D).

6.4.2.C. Weight gain and carcass measurements

There was a significant ($p<0.004$) effect of neonatal handling on body weight, with handled pigs exhibiting lower body weights (Figure 6-8A) and no significant time*treatment interaction. At the time of slaughter neonatally handled pigs weighed significantly ($p<0.05$) less and had significantly ($p<0.02$) higher plasma ACTH concentrations (Figures 33A and B ,respectively). Plasma concentrations of total and free cortisol did not differ between handled and nonhandled boars (Figure 6-8C). Both hot and cold carcass weights (Figure 6-9A) and lean and fat depth (Figure 6-9B) tended to be lower in handled boars; however, these differences were not statistically significant. The percentage lean yield was also not significantly affected by neonatal handling treatment (Figure 6-9C).

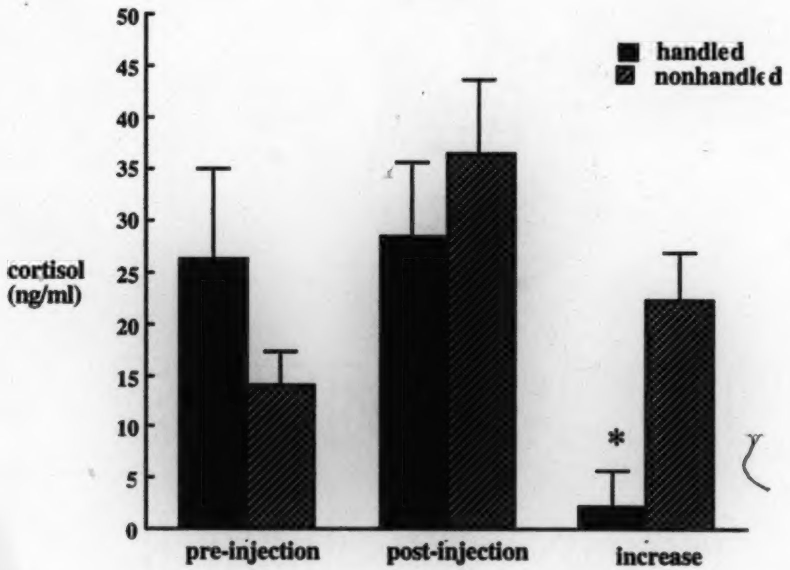

Figure 6-1. Neonatal handling induced a cortisol release which resulted in a blunted response to Ketamine injection. Plasma cortisol concentrations in handled and nonhandled (n=6 for each treatment) 14 day old pigs prior to anaesthesia with Ketamine (pre-injection), at the time of decapitation (post-injection), and the amount of cortisol increase between pre and post values. *significantly different at p<0.0001.

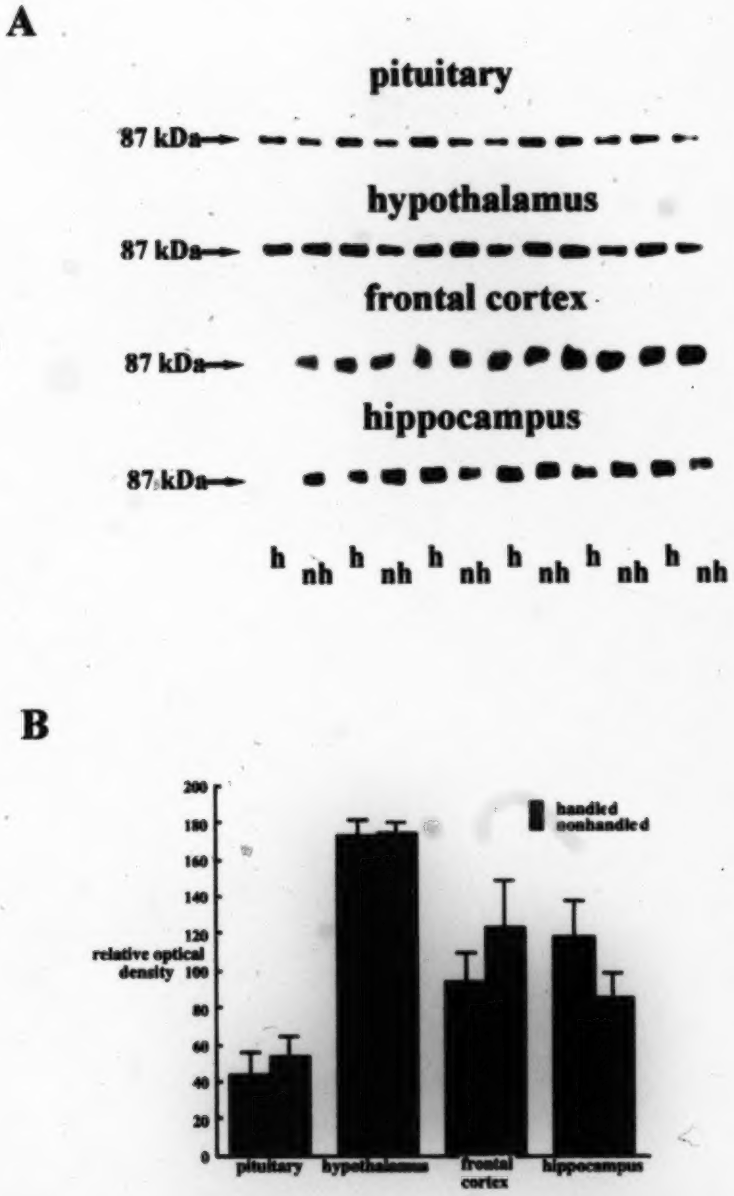

Figure 6-2. Neonatal handling did not effect immunoreactive brain GRs. A: representative western blots performed on tissue from 12 (n=6 per treatment) fourteen day old pigs subjected to neonatal handling (h) or nonhandled controls (nh). B: graphical representation of optical densitometry results.

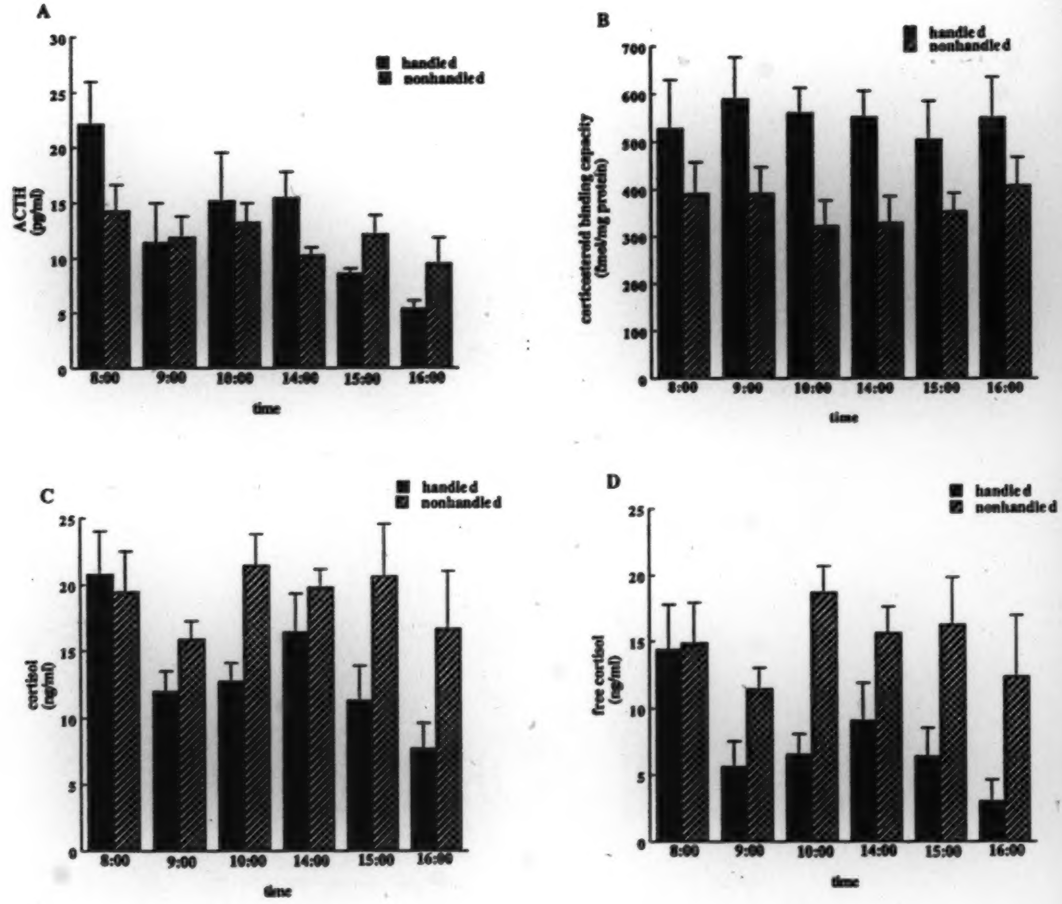

Figure 6-3. Neonatal handling significantly increased CBC and decreased nadir basal plasma total and free cortisol in adult boars. Data for 5 neonatally handled pigs and 8 nonhandled control pigs. A: basal plasma ACTH concentrations. B: plasma corticosteroid binding capacity (CBC). C: basal total cortisol concentrations. D: basal free cortisol concentrations estimated from CBC values. *significantly different at $p<0.05$. **significantly different at $p<0.01$.

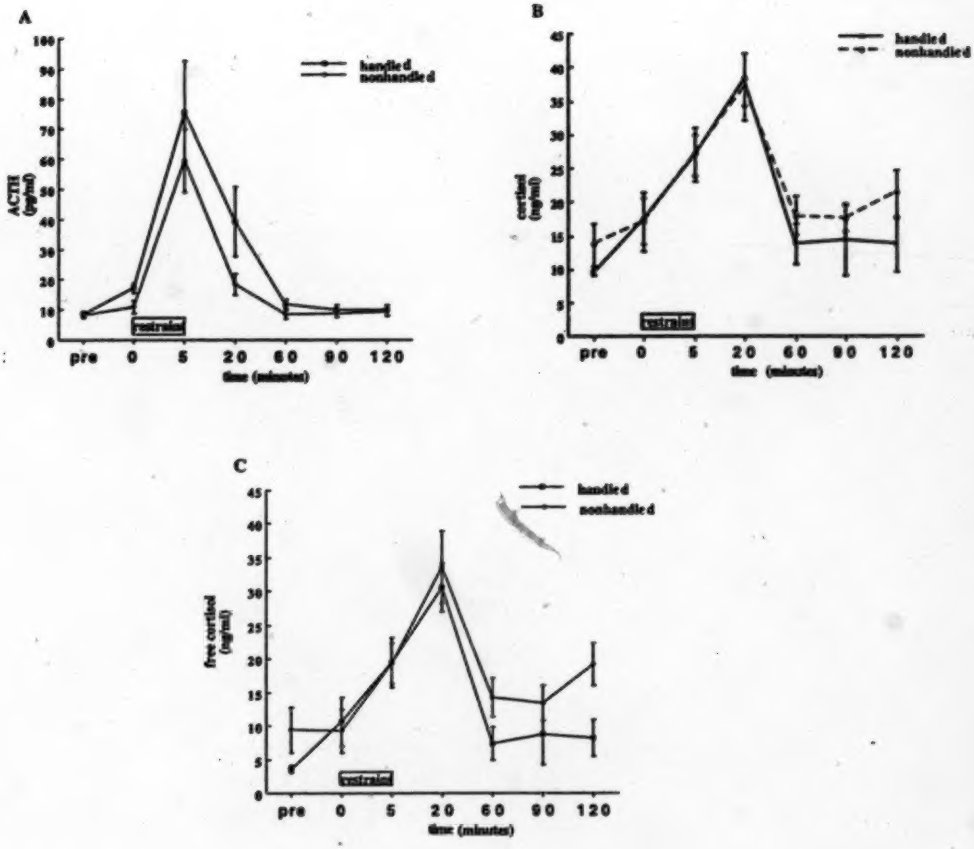

Figure 6-4. ACTH response to a 5 minute application of a nose snare was increased in neonatally handled boars with no significant difference in total or free cortisol concentrations during peak response. Data for 5 handled and 8 nonhandled pigs. A: plasma ACTH. B: plasma total cortisol. C: estimated free cortisol. *significantly different at p<0.05.

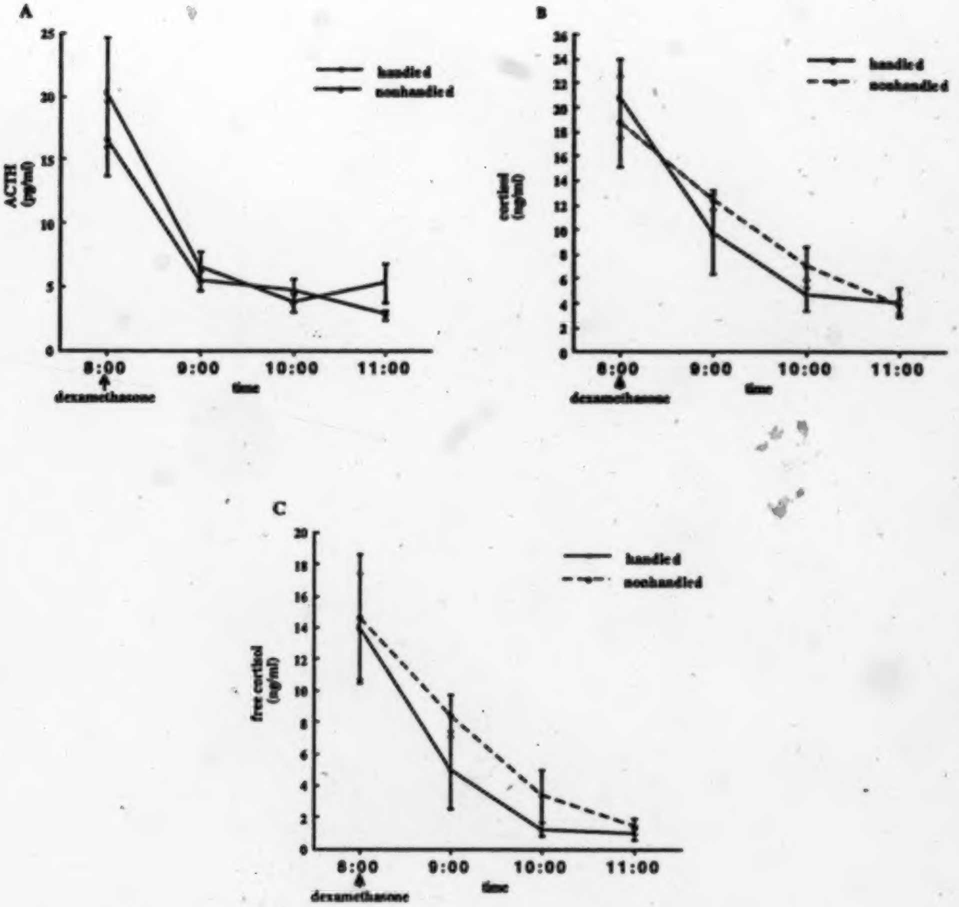

Figure 6-5. Dexamethasone suppression of ACTH and cortisol did not differ between neonatally handled and nonhandled pigs as adults. Data for 4 handled and 7 nonhandled pigs. 8:00 sample refers to sample obtained from basal testing. A: plasma ACTH. B: plasma total cortisol. C: estimated free cortisol.

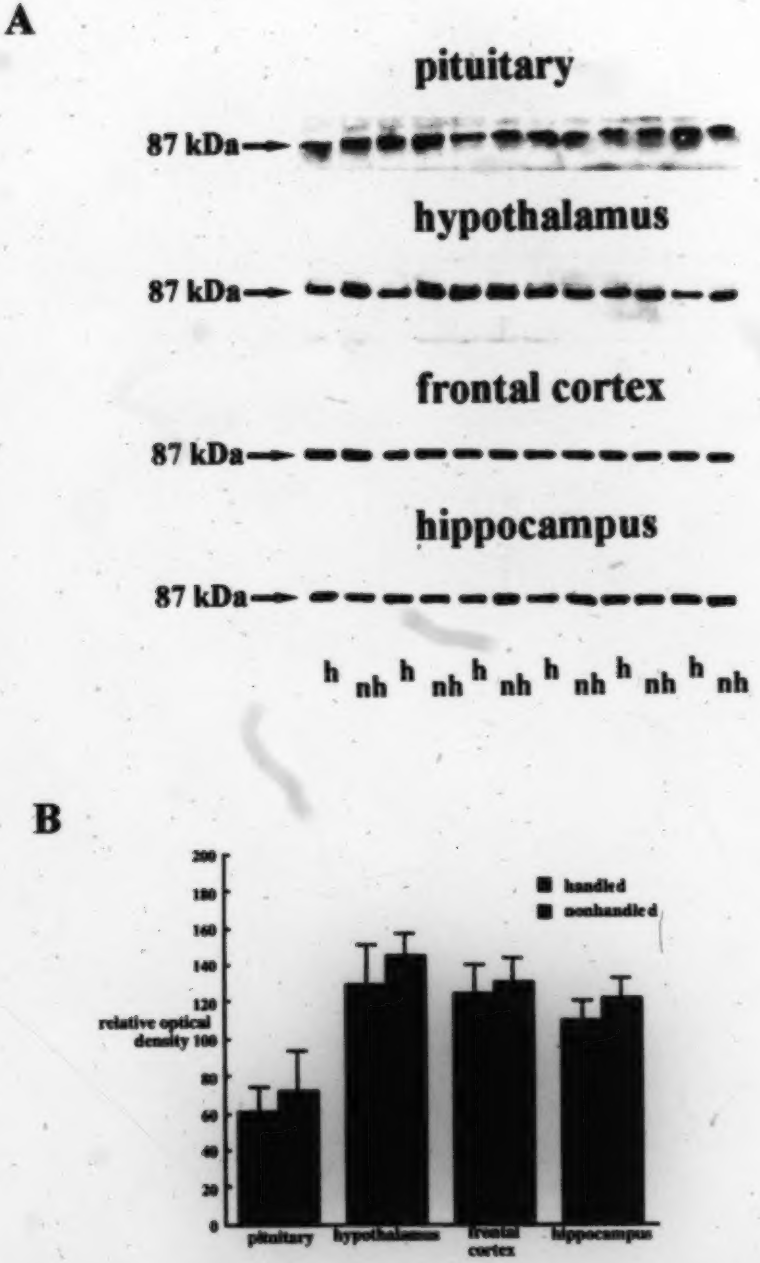

Figure 6-6. Neonatal handling did not alter immunoreactive GR expression in brain tissue from adult pigs. A: representative western blots performed on tissue from 7 month old pigs subjected to neonatal handling (n) (n=6) or nonhandled controls (nh) (n=7) B: graphical representation of optical densitometry results.

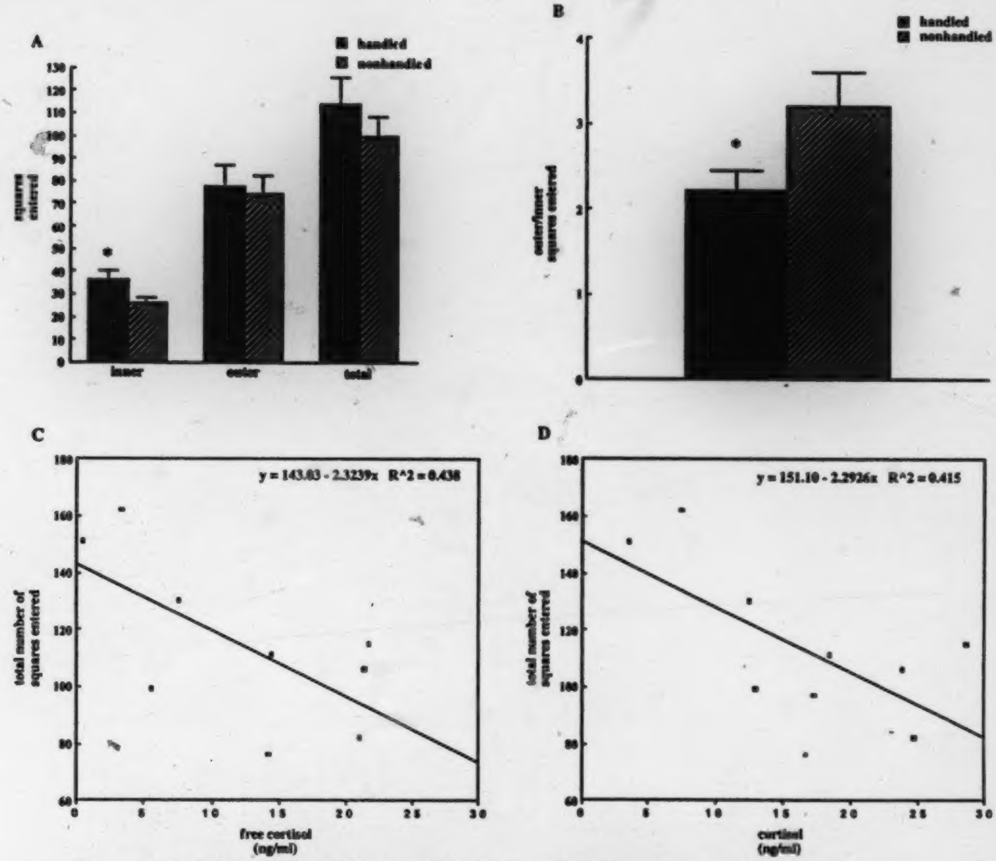

Figure 6-7. Neonatal handling resulted in increased locomotor rates in inner squares in adult boars. Data for 11 handled and 11 nonhandled boars. A: Number of squares entered separated into inner and outer squares with the sum of both as total number of squares entered. B: Ratio of the number of outer entered to inner squares entered. C: Regressions between the total number of squares entered and plasma cortisol concentrations measured at 90 minutes post-snaring during separate stressor testing at a later time. D: Regressions performed with estimated free cortisol from the same test as described above. Both regressions were statistically significant at $p<0.05$.

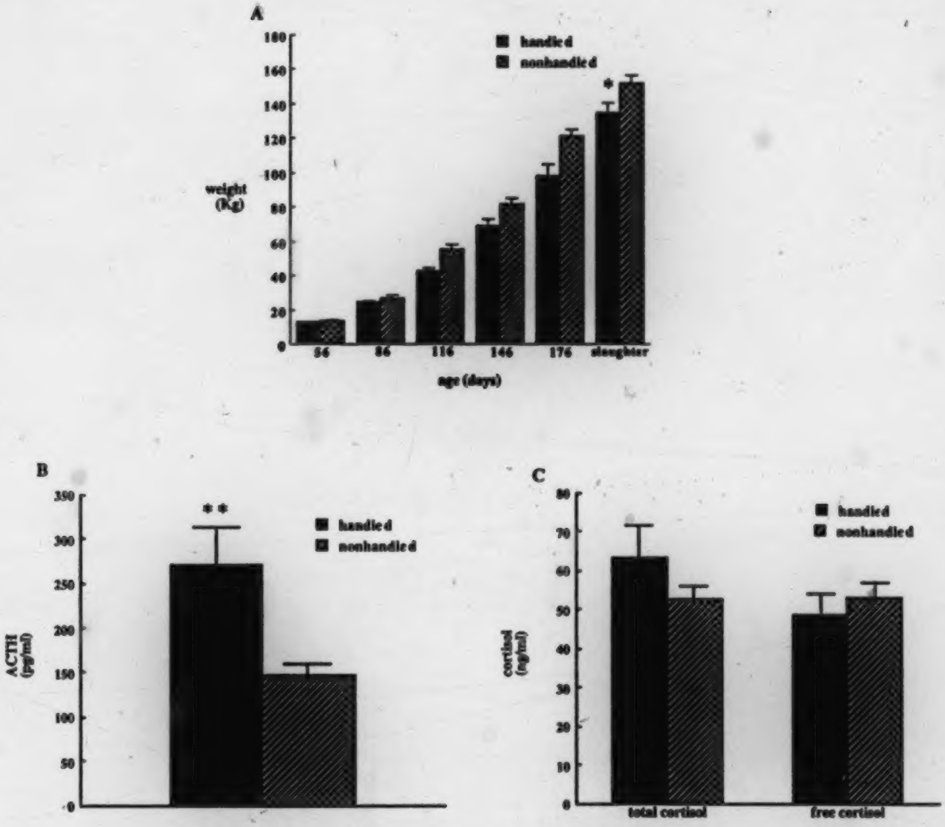

Figure 6-8. Neonatally-handled boars weighed less and secreted significantly more ACTH at the time of slaughter. Data from 11 boars per treatment. A: body weights obtained throughout the 7 months and at the time of slaughter. B: plasma ACTH concentration at the time of slaughter. C: plasma total and estimated free cortisol at the time of slaughter. *significantly different at $p<0.05$. **significantly different at $p<0.02$.

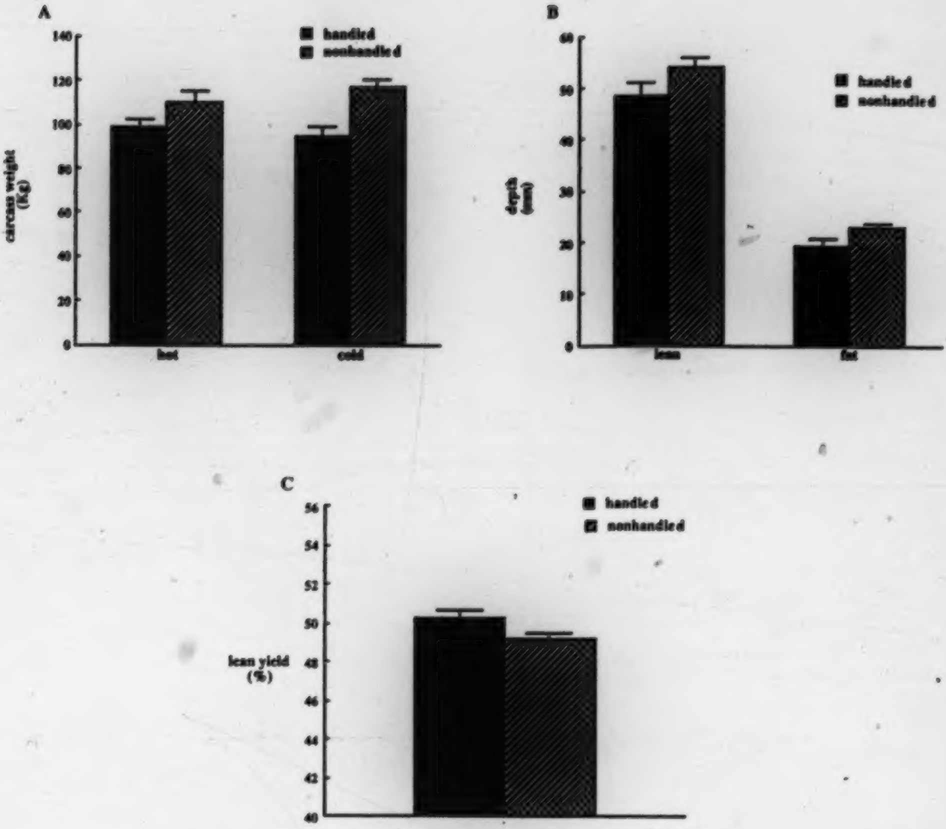

Figure 6-9. No significant differences in carcass measurements were found between handled and nonhandled boars. Data from 11 boars per treatment. A: trimmed carcass weights obtained immediately after slaughter (hot) and 24 hours after slaughter (cold). B: lean and fat depth measurements from Hennessy Probe. C: percentage lean yield obtained from lean and fat depth measurements.

6.5. DISCUSSION

In this chapter the effects of neonatal handling in swine with respect to HPA responsivity and central GR concentrations have been described. Unlike the situation in the rat it was found that neonatal handling did not alter hippocampal GR concentrations. However, handling did alter HPA responsivity in pigs but in a different manner to that found in rats.

6.5.1. Neonates

The neonatal paradigm applied to pigs for 10 minutes per day for the first 14 days of life induced a cortisol release in response to handling. This is surmised based on the trend towards higher cortisol concentrations in handled pigs 30 minutes post-handling and the attenuated response of handled pigs to the second stressor of Ketamine injection indicated by a significantly lower increment in plasma cortisol (Figure 6-1). Keller-Wood and Dallman (1984) reported that when stressors are separated by less than 2 hours there is a dampening of the corticosterone response to the second stressor in rats. Differential responses of the pigs to being handled at the time of injection may also result from a habituation to handling.

The developmental effects of cortisol in pigs is not known. Rat pups injected with 100 μg corticosterone for the first 2 weeks of life had no difference in GR receptor concentrations when examined at 70 days of age (Meaney et al. 1985), therefore, the elicitation of cortisol release as a function of handling would not be expected to confound results in rats.

Neonatal handling had no effect on immunoreactive GR concentrations in any brain region examined (Figures 6-2A and B), unlike the situation in rats where an upregulation of hippocampal GR occurred which was detectable by as early as day 7 (Meaney and Aitken 1985). In the current work a detergent extraction method was used which would not liberate nuclear GRs into the soluble cytosolic fraction in neonatal pigs. The differential stress responses to Ketamine injection at the time of sacrifice may confound our results in that higher concentrations of GR may be translocated to the nucleus at the time of slaughter in handled pigs.

6.5.2. Adults

Even though changes in central GR concentrations at day 14 were not seen, HPA function and central GR concentrations were examined in the adult animals. At the time of weaning it was noticed that dramatic differences existed in the behaviour of handled pigs, in that entry into their pens evoked no response in the handled pigs, whereas entry into pens containing nonhandled pigs caused the pigs to run towards the back of the pen in order to avoid the experimenter. It was hypothesized that a "different" pig had been produced and subsequent analysis determined if these differences were permanent and whether they would result in differential HPA function in adults.

6.5.2.A. HPA function

Handled pigs had a trend towards higher peak ACTH reflected in the 8:00 basal sample, in their responses to restraint, and in the ACTH concentrations measured at slaughter which were significantly higher (Figures 6-3A, 6-4A and 6-8B respectively). The basal sampling did not include sufficient time points during peak activity. Restraint stress produced only a mild HPA response. This was possibly due to it being the second time the boars were exposed to snaring as Klemcke (1994) has shown an attenuated response five days after the first exposure. Under restraint stress the handled boars mounted an ACTH response faster and were slower to terminate the response. By contrast, peak cortisol (total or free) concentrations, either basally or stressor-induced, did not differ as a function of neonatal treatments (Figures 6-3B, 6-4B and 6-8C) which suggested some degree of adrenal insensitivity in neonatally handled pigs.

Nadir concentrations of ACTH during basal sampling did not differ between treatments with the exception of one time point (14:00) ,while total and free cortisol concentrations were significantly reduced in handled boars (Figure 6-3). During the recovery phase of stressor testing, handled boars had significantly lower free cortisol 2 hours after stressor-termination but this appeared to be reflective of the lower basal concentrations during the nadir (120 minute stress recovery sample was taken at 16:00). The nonhandled boars in this experiment appear to have a loss in the circadian rhythm in plasma cortisol concentrations and pigs in restrictive individual housing have been shown to lose this rhythm compared to group housed pigs which is suggestive of a chronic stress response (Barnett et al. 1981, Janssens et al. 1995). The effects of neonatal handling can be summarized as producing boars which exhibited elevated peak ACTH concentrations with no difference in concomitant cortisol and significantly lower nadir total and free cortisol concentrations in the presence of similar ACTH concentrations. The differences in peak ACTH were not a function of feedback differences, presumably via CRH, due to pituitary GR (as evident by a lack of difference in dexamethasone response as well as pituitary GR content) or GR in other brain regions examined. The measure of GR in adults used a whole cell extraction procedure, therefore immunoreactive GR concentrations represent the total population.

The neonatally handled boars resemble the pharmacogenetically apomorphine-susceptible (apo-sus) rats which exhibit an increased stereotypic gnawing response to the dopamine agonist apomorphine compared to apomorphine unsusceptible rats (Cools et al. 1990). These two lines also differ in HPA function and it is the differences in HPA function which appear first, around 3 weeks of age (Rots et al. 1996). Apo-sus rats had increased nadir basal ACTH, no significant differences in nadir total corticosterone but significantly lower nadir free corticosterone concentrations as a function of increased CBG concentrations (Rots et al. 1995). A novel environment stressor elicited a significantly greater and more prolonged ACTH response in apo-sus rats, with no significant difference in free corticosterone concentrations (Rots et al. 1995). Dexamethasone suppression elicited a similar total corticosterone response indicating that GR feedback at the pituitary was not involved in the differences between apo-sus and unsus rats (Rots et al. 1995).

There are 3 primary issues to be discussed with the effects of neonatal handling in pigs and they are; the elevation of CBC, the higher plasma ACTH, and the relative adrenal insensitivity in handled boars which are believed to be all interrelated.

The source of CBC differences seen in neonatally handled pigs is not known; however, it may represent increased synthesis of CBG or albumin, reduced turnover rates of these proteins, or conformational changes which influence binding affinity of these proteins. It was also not determined whether the changes appeared immediately after handling (day 14) or arose later in life in response to other changes in HPA function. Chronic stress or chronic ACTH/corticosterone injections reduced plasma CBG concentrations in rats (Armario et al. 1994, Spencer et al. 1996) and in pigs chronic stress has been shown to decrease (Kattesh et al. 1980) or not affect CBG binding capacity (Klemcke 1994). Neonatally handled pigs had reduced total cortisol concentrations basally; however, whether reductions in cortisol would have the opposite effect on CBG concentrations (resulting in an increase) is not known.

The reduced free cortisol level, resulting from increased CBG concentrations, in conjunction with reductions in total cortisol, may lead to a reduced negative-feedback effect of cortisol on ACTH, presumably via CRH, however this was not measured, and therefore result in elevated ACTH in handled boars. In fetal sheep there is an increase in fetal HPA activity during late gestation without a negative-feedback effect of the increasing glucocorticoid concentration on further release which is proposed to be via increased CBG synthesis (Challis et al. 1995, Challis and Brooks 1989). The blood-brain barrier prevents CBG passage into neural tissue and therefore only non-CBG bound glucocorticoid can pass into the brain (Siiteri et al. 1982) and therefore effect negative feedback. Challis et al. (1995) using pituitary cultures from term fetal lambs, found that increased CBG reduced the negative-feedback effects of exogenous cortisol on ACTH release with no effect of CBG when dexamethasone (does not bind to CBG) was added to cultures. A continuous infusion of cortisol to fetal sheep caused a significant increase in CBG mRNA which is accompanied by a significant increase in plasma ACTH (Challis et al. 1995). The next question was how the differences in nadir cortisol in handled pigs were transduced into elevated peak ACTH, even though there were no differences in nadir ACTH concentrations.

The times of peak circadian ACTH secretion are more sensitive to glucocorticoids than are the times of minimum activity (Dallman et al. 1987).

Treatment of rats with cyanoketone (long acting corticosterone synthesis inhibitor) did not change nadir basal ACTH release but instead resulted in increased basal peak ACTH which maintained a constant average corticosterone concentration over 24 hours (Dallman et al. 1987). In the current work, it its speculated that the significantly lower basal free cortisol observed in handled boars is what drives the trend towards higher peak basal ACTH at 8:00.

Since corticosteroid feedback depends on central drive, plasma free corticosteroid, and functioning of brain corticosteroid receptors, it is not possible to determine whether the trend towards elevated ACTH response to the restraint stressor ,and significantly higher ACTH response at slaughter, resulted solely from reduced basal nadir free cortisol. Stressors applied during nadir periods (restraint stress was applied in the afternoon and pigs were slaughtered in the late morning), when free cortisol concentrations are low, are being applied upon a period of reduced tonic negative feedback of cortisol on ACTH, presumably via CRH, which may result in a relatively higher drive on ACTH release from CRH upon stressor application at this time. Significantly higher ACTH concentrations were detected at the onset of stress which tends to suggest a lack of tonic negative feedback on drives to ACTH release most likely via CRH. Differences in central GR receptors may be excluded as playing a role in the reduced negative-feedback on further ACTH release, since no differences in these receptors have been shown in any brain region examined. However, this does not exclude the possibility of differences in other brain structures.

In the presence of higher concentrations of ACTH handled boars did not differ in total cortisol release during restraint stress or slaughter which suggests adrenal insensitivity. This is also supported by the lower nadir cortisol in handled boars in the presence of ACTH concentrations similar to nonhandled boars. In apo-sus rats, much higher concentrations of ACTH are required in order to reach an equivalent corticosterone level to that found in apo-unsus rats and therefore apo-sus rats also exhibited adrenal insensitivity (Rots et al. 1995). Exogenous ACTH administration, in dexamethasone-suppressed apo-sus and unsus rats, elicited a similar total corticosterone response (Rots et al. 1995). Rots et al. (1995) have proposed that the adrenal hyporesponsivness does not result from differential sensitivity to ACTH but rather differences in norepinephrine stimulation of corticosterone release as apo-sus rats have been found to have 30% lower adrenal norepinephrine content. The lack of difference in cortisol concentrations in the presence of increased ACTH in handled boars may also reflect changes in cortisol metabolism and not synthesis or release. The source of adrenal insensitivity in neonatally handled boars remains to be determined.

6.5.2.B. Behaviour

Neonatally handled boars had higher locomotion scores in inner squares in the open field test (Figure 6-7A). Open field tests have been used to measure animal behaviour, specifically emotionality (behavioral changes assumed to result from increased sympathetic activation) (Archer 1973, Beilharz and Cox 1967). It has also been used to provide a measure of reactivity of an animal when placed in an unfamiliar environment and as a measure of temperament or excitability (Fraser 1974, Kilgour 1975, von Borell and Hurnik 1991). de Passille et al. (1995) reported that different behaviours in calves in an open field test reflect different motivations with sniffing and licking being exploratory behaviours, defecation and vocalization representing fearful responses, and running and jumping being reflective of the first time animals are let loose in a large open area. The current data has been interpreted as indicating that handled boars are more willing to enter into the center of a large open area which is novel to them despite the presence of an unfamiliar noise stressor (suspended radio). This is not a reflection of increased total mobility (not significantly different) as the ratio of outer squares entered per inner square was significantly lower in handled boars, indicating that a larger proportion of their ambulations were spent in the inner squares. The analysis of patterns of locomotion (inner versus outer squares) represents a significant improvement in open field testing where increased locomotion over inner squares has been shown to be indicative of reduced anxiety (Ramos and Mormede 1998). The results are not expected to be confounded by individuals prior experience with humans during the handling procedure as Hemsworth and Barnett (1992) have demonstrated that the reduced fear responses to humans generated by their particular neonatal handling treatments were absent by 24 weeks of age. In addition, if the handled pigs, which exhibited possible reductions in anxiety, were accustomed to humans they should show reduced ACTH responses to stressor which in fact they did not. These data could be interpreted as a decrease in fearfulness; however, ascribing an emotional difference between neonatally handled and nonhandled boars on the basis of one behavioural test would be inappropriate. It does suggest that a wider battery of tests would be worthwhile in order to determine how these animals differ behaviourally.

The open field test represents a novel environment stressor in pigs and one which will induce HPA activation (Mormede et al. 1984, Desautes et al. 1997). A significant negative correlation has been found between the total number of squares entered and total and free cortisol measured 90 minutes post-termination of restraint stressor (r=.643 and r=.652 respectively) (Figures 6-7\C and D) which was found irrespective of treatment. von Borell and Ladewig (1992) found a significant positive correlation between adrenocortical reactivity, to exogenous ACTH (0.0025 IU/kg) and ambulation (r=0.33, P<0.01) and vocalization (r=0.37, P<0.05) in open field testing of pigs which was consistent over testing at 10 or 28 weeks of age. Desautes et al. (1997) found no significant correlation between cortisol concentrations prior to open field testing or immediately after with total mobility or vocalisations in an open field test. The authors have suggested that there is no biological link between behavioral reactivity and the adrenocortical axis.

The difference between results described in this thesis and those reported above is that the cortisol concentrations von Borell used and the post-open field test cortisol concentrations in Desautes's work were approximately 10 times higher than the 90 minute post-stressor termination cortisol concentrations reported in the current work. It was found that the 90 minute post-stressor cortisol concentrations (still above basal secretory concentrations) were the only concentrations that correlated with mobility scores in the present study. The "basal" concentrations reported by Desautes's group are approximately 3 fold higher than the elevated cortisol concentrations at 90 minutes post-stressor termination reported in this thesis. It may be that relationships between cortisol and total mobility do not exist over all concentrations of elevated cortisol and that other factors may play more important roles. Alternatively, in the case of von Borell and Ladewig's work, the effects of relatively high plasma cortisol may have the opposite effects on ambulation as that found with relatively lower cortisol concentrations. In fact MR activation (low levels of plasma corticosterone) increased exploration of novel objects by rats in an open field test pen (Oitzl et al. 1994). In addition, Salakjohnson et al. (1997) have demonstrated that intraventricular administration of CRH in pigs increased overall activity (ie. Walking) which may indicate that high levels of HPA stimulation result in increased activity which may possibly be reflected in total locomotion in an open field test pen. What is of interest in the current findings, is that cortisol concentrations were not correlated with the number of inner squares entered, and therefore, was most likely not involved in the behavioural differences between neonatally-handled and -nonhandled boars.

6.5.2.C. Weight gain and carcass measurements

The consequences of neonatal handling appeared to be a tendency towards reduced growth rates with significantly lower body weights detected on day 56, 116, and at slaughter (Figure 6-8A). Replacement of corticosterone at varying doses in ADX rats has revealed a biphasic dose-response curve for body weight gain with low doses resulting in lower body weight and progressively higher doses resulting in increased body weight up to the point where higher corticosterone concentrations start to cause a catabolic effect resulting in a suppression of weight gain (Akanaet al. 1985, Devenport et al. 1989). The increasing body weights associated with increasing corticosterone were associated with MR occupation while catabolic effects were associated with GR occupation (Devenport et al. 1989, Devenport and Smith 1992). In fact, aldosterone administered to ADX rats increased weight gains while the specific GR agonist RU28362 resulted in reduced body weight (Devenport et al. 1989, Devenport and Smith 1992). Fat: lean ratios were found to increase linearly with corticosterone replacement level in ADX rats (Devenport et al.

1989). It can be concluded that the lower body weights and trend towards leaner carcasses may have resulted from the lower basal nadir cortisol concentrations in handled pigs.

6.5.3. Summary

Neonatal handling in pigs did provide permanent changes in HPA function although these changes were different from the results observed in rats. The handling procedure induced a cortisol release in neonates which resulted in a blunted cortisol response to a second stressor of Ketamine injection. At 14 days of age no differences in GRs were detected in any brain region examined. As adults, neonatally handled boars had higher peak ACTH with no difference in cortisol. Nadir total and free cortisol concentrations were significantly lower in handled boars and CBC concentrations were significantly elevated. The differences in HPA function between treatments were not reflected in differences in brain GR concentrations but behaviourally handled boars could be distinguished from nonhandled boars by increased locomotion in the inner squares of an open field test. In spite of what appeared to be an advantageous basal HPA function, handled boars had lower growth rates and were significantly lighter at the time of slaughter. It can be concluded that there is a sensitive period during early postnatal development in the pig where environmental manipulations can result in permanent changes in HPA function.

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CHAPTER 7

THE EFFECTS OF MUTATED SKELETAL RYANODINE RECEPTOR ON HPA FUNCTION IN PIGS

7.1. INTRODUCTION

Having examined the effects of early neonatal environment on future HPA function in pigs, attention was then turned to investigating HPA function in pigs with a stress-susceptible phenotype possessing a mutation in skeletal ryanondine receptors (sRyR) which is known to cause porcine stress syndrome (PSS) (Fujii et al. 1991). PSS refers to the heritable condition characterized by excessive calcium flux in skeletal muscle and uncontrolled contraction upon exposure to stressors (MacLennan and Philips 1992). At the time of slaughter, pigs with PSS exhibit a rapid decrease in muscle pH while the carcass temperature is still relatively high which results in denaturation of muscle proteins and associated water loss which gives the meat a pale soft exudative (PSE) appearance (MacLennan and Philips 1992). The susceptibility to inducing the syndrome upon exposure to stressors suggests that these animals may also show altered stress responses.

In the past, studies of HPA function in pigs have described animals which showed an enhanced cortisol response to exogenous ACTH and these pigs are referred to as "stress susceptible" (Marple et al. 1972, Sebranek et al. 1973). The HPA data obtained from "stress susceptible" pigs has often been cited as supportive data for research on PSS pigs (Roberts et al. 1996, D'Allaire and DeRoth 1986, Aberle, et al. 1976, Schaefer et al. 1990, Nyberg et al. 1988). In the absence of halothane testing, or the more recently available genotyping methods, "stress susceptible" pigs are not necessarily representative of PSS pigs.

This chapter addresses the question of whether pigs possessing mutated sRyR do in fact exhibit altered HPA function and what is the nature of these differences. The response in heterozygous animals has been examined since 19% of pigs tested from Canadian farms were heterozygous for the mutation and as such were a quantitatively more important group than the homozygous animals which represent only 0.7% of the pig population (O'Brien et al. 1993).

7.2. METHODS

7.2.1. Animals

All experminets were performed under the approval and guidelines of the Canadian Council on Animal Care. Two genotypes of male pigs were used for this experiment: those animals which were heterozygous (n=10) for the nucleotide 1843 substitution for sRyR derived from a Pietrain-National Pig Development cross, and wild-type (n=11) animals which were National Pig Development pigs not exhibiting a mutated skeletal ryanodine receptor. The wild-type pigs used in this experiment were the nonhandled controls from Chapter 6 as the experiments in this chapter were performed concurrently with the handling experiment discussed in Chapter 6. The nonhandled controls in Chapter 6 were negative for the mutation in sRyR and therefore function as controls for this chapter. Testing of males was performed at the same age (7 months) which entailed animals being tested on different days. Treatments groups were tested on different days but were interspersed with each other.

7.2.2. Open field test

At 7 months of age boars were behaviourally tested for treatment effects using the open field test. The open field test was performed in a 9 m x 4 m concrete floored area enclosed with plywood. The area was marked off into 72 equal sized squares (0.5 m^2) using black spray paint with the innermost 16 squares scored as "inner". A radio was suspended above the inner area and was used as a noise stressor. The entrance door was continuous with the plywood sheeting and a viewing window was present at one end of the pen to enable scoring of mobility. A 1 minute adjustment period was given once the boar entered the pen and mobility was recorded for 5 minutes after adjustment. The number of inner and outer squares entered by the left foreleg was recorded. Testing was performed on boars just prior to moving them into farrowing crates.

7.2.3. Catheterization and blood sampling

Boars were housed in farrowing crates for 4 or 5 days prior to catheterization in order to allow the animals to adjust to confinement. The rear of the line of crates was covered with black plastic in order to provide a visible barrier between the back alleyway where the individual sampling would be located. This was an effective barrier in that if the sampler lifted the black plastic the pigs were noticeably startled. Indwelling ear vein catheters (Cidex sterilized (Surgikos Canada) PE 90 0.8mm i.d., 1.2 mm o.d., Intramedic tubing, Becton Dickinson) were implanted 20-30 cm, under aseptic conditions, in snared boars 4 days prior to sampling. Catheter patency was maintained by leaving the lines filled with heparinized saline. Catheters were not flushed between sampling periods. Extension lines (220 cm of PE 90 tubing with a 16 guage needle blunted on both ends as a connector) were added to the catheter lines one hour prior to sampling and exteriorized through a small hole in the rear blind and heparinized saline was removed and the catheters were flushed with saline. For each sample 2 mls of blood were collected on ice in EDTA-coated 6 ml polypropylene tubes containing 50 µl saturated EDTA and 500 UIK Trasylol (in 50 µl). Hypovolemia was prevented by replacing 2 mls of saline after each sample was obtained and the catheter line was left filled with saline. Blood samples were stored on ice in a refrigerator for no longer than 3 hours and then centrifuged at 3,000 RPM at 4^0C for 15 minutes. Plasma was stored at -80^0C until analysis.

7.2.4. Basal blood sampling

Five days after catherterization basal blood sampling was performed in undisturbed boars. Boars were fed *ad libitum* at 6:00 and catheter extensions were attached. Sampling times were 8:00, 9:00, 10:00, 14:00, 15:00, and 16:00. During this sampling period no one entered the barn and the sampler remained behind the blind.

7.2.5. Stressor testing

Two days after basal samples were obtained boars were stressor tested. Catheter extensions were attached at 12:00 and a pre-stressor sample was taken at 13:00. Boars to be stressor-tested on a given day were crated adjacent to each other and nose-snared for 5 minutes at the same time. Blood samples for the onset and end of restraint were obtained and the barn was vacated. Recovery samples were obtained from the extension lines behind the blind. Samples were obtained 20, 60, 90, and 120 minutes post-termination of snaring.

7.2.6. Dexamethasone suppression

Two days after the completion of stressor testing boars were treated with Dexamethasone. Boars were fed *ad libitum* at 6:00 and catheter extensions were attached. Boars were injected with 0.04 mg/kg Dexamethasone (Sebranek 1973) (suppresses ACTH and cortisol release by binding to GR receptors) via the extension line at 8:00 and the drug was delivered via flushing the line with 5 mls of saline. Blood samples were obtained from behind the blind at 9:00, 10:00, and 11:00 with no one entering the barn from 7:00 until testing was completed.

7.2.7. Euthanasia of adult boars

Four days after completion of dexamethasone-suppression testing, boars were returned to the grower/finisher area and housed singly until euthanasia 5 days later. Boars were transported to an on-site meat facility at 6:00 and all animals were sacrificed by 10:00. Live weights were obtained and the animals were electrically stunned and exsanguinated. Craniotomies were performed using a hand held circular saw and the brains were collected along with the pituitary and snap frozen in liquid nitrogen and stored at -80^{0}C. Blood was collected from the stick wound in polypropylene EDTA coated tubes containing 100 μl of saturated EDTA and 1000 UIK Trasylol (100 μl) for plasma cortisol and ACTH measurement. Whole blood was also collected in an EDTA coated vacutainer (Becton Dickenson) and frozen at -20^0 C until analysis for genotype determination.

7.2.8. Carcass evaluation

Live animal weights were recorded as well as trimmed hot and cold weights (24 hours *post mortem*) of sides. Lean and fat depths were obtained using a Hennessy grading probe, placed between the last 2 ribs, and a predicted percentage lean yield was obtained. *Longissimus dorsi* muscle pH and temperature were measured 1 hour post mortem and pH was measured again 24 hours later. Objective colour measurements were obtained 48 hours *post mortem* using one surface-measurement of reflectance for a 25 mm slice of the longissimus dorsi muscle using a Chroma Meter II (Minolta Canada Inc. Mississauga, Ont. CA.) . Subjective structure scores were also obtained at this time by 3 independent graders. Fluid drip loss from a 100 g *longissimus dorsi* muscle sample was determined over 48 hours and expressed as grams lost per 100 g.

7.2.9. Genotype determination

Detection of the presence of mutated sRyR was performed with a modification of the procedure reported by Otsu et al. (1992). Genomic DNA was extracted from 100 µl of whole blood with 500 µl of hypotonic DNA extraction buffer (10 mM Tris and 1 mM EDTA) and centrifuged at 1,100g for 2 minutes. After 3 washes with the extraction buffer, proteins in the pellet were digested with 100 mg/ml proteinase K (in 50 mM KCl, 10 mM Tris, 5% (v/v) Tween 20) with incubation at 56^0C for 1 hour. Proteinase K was inactivated by boiling for 10 minutes. Samples were centrifuged at 12000 rpm for 2 minutes and the supernatant containing the DNA was used for Polymerase Chain Reaction (PCR) amplification of sequences containing the skeletal ryanodine receptor. Forward primer sequence (5^1 - TCC AGT TTG

CCA CAG GTC CTA CCA - 3^{I}) and reverse primers (5^{I} - ATT CAC CGG AGT GGA GTC TCT GAG - 3^{I}) corresponding to intronic sequences flanking the exon of interest, were used to amplify a 659 bp sequence which contained the site of a possible thymine for cytosine substitution at bp 1843 for sRyR. This single base change creates a BsiHKA restriction site in carrier animals along with another common BsiHKA site found in both mutated and wild type DNA as a control. The PCR mix contained 1 mM of dATP, dGTP, dTTP, dCTP each (Perkin Elmer Cetus), 2 mM $MgCl_2$ (Perkin Elmer Cetus), 0.1 μM of each primer, 0.75 units of Taq DNA Polymerase (Promega), GeneAmp 10X PCR buffer II (Perkin Elmer Cetus) (final concentrations: 10 mM Tris-HCl pH 8.3, 50 mM KCl). The PCR program was 94^{0}C for 1 minute followed by 65^{0}C for 1 minute repeated for 35 cycles with a final extension time of 7 minutes at 72^{0}C. Restriction enzyme digestion was performed with 5 units of BsiHKA I (Promega) in NEB buffer (Promega) at 56^{0}C for 1 hour. Digested samples were electrophoresed on 8% (v/v) polyacrylamide gels with Tris-borate electrophoresis buffer for 2.5 hours, stained with ethidium bromide (0.5 μg/ml) and photographed using a Fisher Biotech polaroid camera with Polaroid Type 667 film and Wratten orange #15 filter over UV light. Carrier animals were detected by the presence of bands at 524, 358, 166, and 135 bp whilst the normal animals only showed bands at 524 and 135 bp (Figure 7-1).

7.2.10. Plasma corticosteroid-binding globulin assays

Plasma corticosteroid binding capacity (CBC) was measured using a method previously described by Martin et al. (1977). Samples used were those collected on the basal sampling day and therefore 6 samples for each animal collected at 8:00, 9:00, 10:00, 14:00, 15:00, and 16:00 were assayed. Endogenous steroid was removed from the plasma by passing a 20 µl aliquot through a 10 cm x 1 cm Sephadex LH-20 column and the plasma was diluted 50:1 with TEDGM (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate, pH 7.4). Diluted plasma (225 µl) was incubated with 150 µl of TEDGM containing a previously determined (Martin et al. 1977) saturating 80 nM concentration of [^{3}H]cortisol for 90 minutes at 4^{0}C. Non-specific binding was determined in parallel using a 200-fold excess of cold cortisol. Bound cortisol was separated from free cortisol using 10 cm x 1 cm Sephadex LH-20 columns in triplicate for both the specific and non-specific incubations. The bound cortisol was eluted with 500µl of TEDGM into mini-vials into which 5 mls of Ecolite scintillation cocktail was added and the [^{3}H]cortisol was counted with a Beckman scintillation counter at 45% efficiency for 5 minutes per vial. Protein content in the diluted stripped plasma was determined by the method of Bradford (Bradford 1976) using BioRad Bradford dye and results were expressed as picomoles binding/mg of protein.

7.2.11. Radioimmunoassays

Plasma cortisol concentrations were measured with GammaCoat™ Cortisol ^{125}I RIA Kit (Incstar Co.) using 100 µl of plasma. This kit has previously been validated for use with porcine plasma samples (Cook et al. 1996). The detection limit of the assay was 2 ng/ml and intra- and inter-assay coefficients of variability were 8% and 10%, respectively.

Plasma ACTH was measured by the radioimmunoassay described by Walker et al. (1990) using a specific antiserum at a final dilution of 1:120,000 (IgG Corp.), [^{125}I] ACTH (Incstar), and 200 µl of plasma sample. The ACTH antiserum crossreacts 100% with $ACTH_{1-39}$, $ACTH_{1-18}$, and $ACTH_{1-24}$, but less than 1% with $ACTH_{1-16}$, ß-endorphin, α- and ß-MSH and α- and ß-lipotropin. Plasma samples were incubated for 48 hours at 4^0C with antiserum and tracer, then goat anti-rabbit IGg (Peninsula Laboratories) was added and incubated overnight. Bound peptide was obtained by centrifugation at 5000g for 45 minutes. The detection limits of the assay were 1 pg/ml. Intra- and inter-assay variability was 8% and 15%, respectively.

7.2.12. Whole cell extract western blotting for GR

The hypothalamus, frontal cortex, and hippocampus was dissected from frozen whole brains post-freezing from a randomly chosen subset of pigs (n=8 heterozygous and n=7 wild-type). Brain tissue was allowed to come to approximately -20^0 C and tissue was dissected using ice-cooled tools. Tissue samples were then powderized in liquid nitrogen using a pestle and mortar. For whole cell extract preparations, the 100 µl of powderized tissue samples were homogenized on ice using a Vibra Cell sonicator in 3 volumes of cold TEDGM (30mM Tris, 1mM EDTA, 1mM dithiothreitol, 10% (v/v) glycerol, 10mM molybdate, pH 7.4) containing 0.4 M NaCl and protease inhibitors (aprotinin (3.5µg/ml), AEBSF (0.4 mg/ml), leupeptin (1 µg/ml) and pepstatin (1µg/ml)). The homogenates were centrifuged at 100,000g for 45 minutes at 4°C in a Beckman Ultracentrifuge. The supernatant was collected on ice and frozen at -80^0C. Protein concentrations were determined using the Bradford assay (Bradford 1976). 50 µg of protein per sample was added to a commercially available (Novex) 4X sample buffer (1.17 M sucrose, 563 mM tris base, 423 mM Tris-HCl, 278 mM SDS, 2.05 mM EDTA, 0.88 mM Serva Blue G250, 0.70 mM Phenol Red) with 10% (w/v) dithiothreitol added just prior to denaturing samples at 70^0C for 10 minutes. Samples were electrophoretically separated in a Novex minigel apparatus (Helixx Technologies) in at least duplicate. Precast 4-12% Bis-Tris NuPAGE gels and a MOPS SDS electrophoresis buffer (1 M 3-(N-morpholino) propane sulfonic acid, 1 M tris base, 10% sodium dodecyl sulfate, 20.5 mM EDTA) were used along with 0.25% (v/v) antioxidant which was purchased from Novex and added to the inner chamber electrophoresis buffer. Proteins were transferred to PVDF membranes using the BioRad wet transfer apparatus with a modified version of the recommended NuPAGE transfer buffer (25 mM Tris base, 25 mM bicine, 1 mM EDTA, 0.03% (w/v) sodium dodecyl sulfate, 20% (v/v) methanol, and 0.1% (v/v) antioxidant purchased from Novex and added immediately prior to transfer). Membranes were blocked with 5% (w/v) skim milk powder for 45 minutes and washed. A 1: 500 dilution of the primary antibody (anti-GR antibody #51 from Affinity BioReagents, N.J., U.S.A.) in TBS-T (20 mM Tris base, 140 mM NaCl and 0.01% (v/v) Tween 20) containing 0.5% (w/v) skim milk powder was incubated with the membrane overnight at 4^0C. The membrane was washed with 3 changes of TBST after which a 1: 5000 dilution of the anti-rabbit horseradish peroxidase linked secondary antibody in TBS-T containing 0.5% (w/v) skim milk powder was incubated with the membrane for 1 hour at room temperature. Hyperfilms (Amersham, Toronto, Ont. CA.) were exposed to chemiluminescence via a horseradish peroxidase linked secondary antibody and the ECL detection system (Amersham, Toronto, Ont. CA.). The intensity of bands were analyzed using relative optical densities determined using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catharines, Ontario). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent protein loading.

7.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the Super Anova Statsview software package from Abacus Concepts. Data presentation is in the form of means with standard errors of the means. Plasma cortisol, ACTH and CBC data obtained from basal samples, stressor testing, and dexamethasone suppression tests, as well as body weights, were analyzed using 1 factor ANOVA with a repeated measures factor (time of sample) within each treatment and treatment as the between factor followed by *post hoc* tests. A 50% catheter failure rate occurred with remote sampling using ear vein catheters, therefore, the number of animals that plasma samples were obtained for during each test is reported at the bottom of the respective graphs. Open field test data and carcass data and slaughter weights were analyzed using unpaired Students t test as insufficient pigs representing the litters were available to test the effects of litters. Correlation analysis and simple regressions were used to analyze relationships between plasma cortisol and open field test mobility scores. Correlational and regression analysis was performed for locomotor behaviour and post-stressor termination plasma cortisol, pituitary GR concentrations and dexamethasone-suppressed plasma cortisol, and hippocampal GR concentrations and peak basal plasma ACTH concentrations on animals in which plasma samples were obtained for each particular test. In addition, insufficient boars representing individual litters were available for analyzing litter*treatment interactions so unfortunately this analysis was not possible.

7.4. RESULTS

7.4.1. HPA function

Genotype had a significant ($p<0.05$) effect on basal ACTH (Figure 7-2A) with heterozygous pigs exhibiting lower plasma ACTH concentrations throughout the sampling times. There was a significant ($p<0.004$) effect of time but not significant time*genotype interaction. There was also a significant ($p<0.008$) effect of genotype on basal cortisol concentrations with heterozygous animals exhibiting lower plasma cortisol (Figures 7-2C). There was no significant effect of time, however, there was a significant time*genotype interaction in that differences between the two genotypes were greatest during the later time points. The two genotypes did not differ with respect to CBC concentrations (Figure 7-2B).

There were no significant effects of genotype on either plasma ACTH or cortisol in response to a 5 minute snaring stressor (Figures 7-3A and B) with no significant time*genotype interactions. There were also no significant effects of genotype on dexamethasone-suppression of ACTH or cortisol or significant time*genotype interactions (Figures 7-4A and B respectively).

Genotype did not have a significant effect on immunoreactive GR concentrations in any brain region examined (Figures 7-5A and B). Across all boars hippocampal GR concentrations were significantly ($p<0.05$) negatively correlated ($r=-.62$) to peak basal (8:00) plasma ACTH concentrations (Figure 7-6A). Immunoreactive GR in the pituitary gland was significantly negatively correlated ($r=-.60$) to plasma cortisol concentrations 1 hour post-dexamethasone injection (Figure 7-6B).

7.4.2. Behaviour

Genotype had no significant effect on mobility scores in an open field test when analyzed as the number of inner, outer, or total squares entered or as a ratio of outer: inner squares (Figures 7-7A and B). Plasma cortisol at 90 minutes post-stressor termination was significantly ($p<0.05$) correlated to total number of squares entered, however the correlation was in a different direction depending on the genotype with heterozygous pigs showing a positive correlation ($r=.69$) and wild-type pigs showing a negative correlation ($r=-.74$) (Figures 7-7C and D respectively).

7.4.3. Weight gain and carcass measurements

Genotype had no significant effect on body weight throughout the experiment but heterozygous pigs were lighter ($p<0.03$) at the time of slaughter (Figure 7-8A). Plasma ACTH, or plasma cortisol concentrations did not differ between genotypes at the time of slaughter (Figures 7-8B and C). Hot and cold carcass weights did not differ significantly between genotypes, however, *longissimus dorsi* muscle samples from heterozygous pigs had a significantly ($p<0.004$) greater drip loss (Figures 7-9A and B respectively). Lean tissue depth did not differ between genotypes, whilst fat depths were significantly ($p<0.04$) lower in heterozygous pigs which resulted in significantly ($p<0.02$) higher percentage lean yield (Figures 7-9C and D respectively). Carcass temperature at the time of slaughter was significantly ($p<0.04$) higher and carcass pH both at slaughter and 24 hours later was significantly lower ($p<0.03$ and $p<0.003$ respectively) in heterozygous boars (Figures 7-10A and B respectively). Colour brightness (indicative of paler meat) was significantly ($p<0.0004$) higher and subjective structure scores were significantly lower ($p<0.03$) for *longissimus dorsi* slices from heterozygous boars (Figures 7-10C and D respectively).

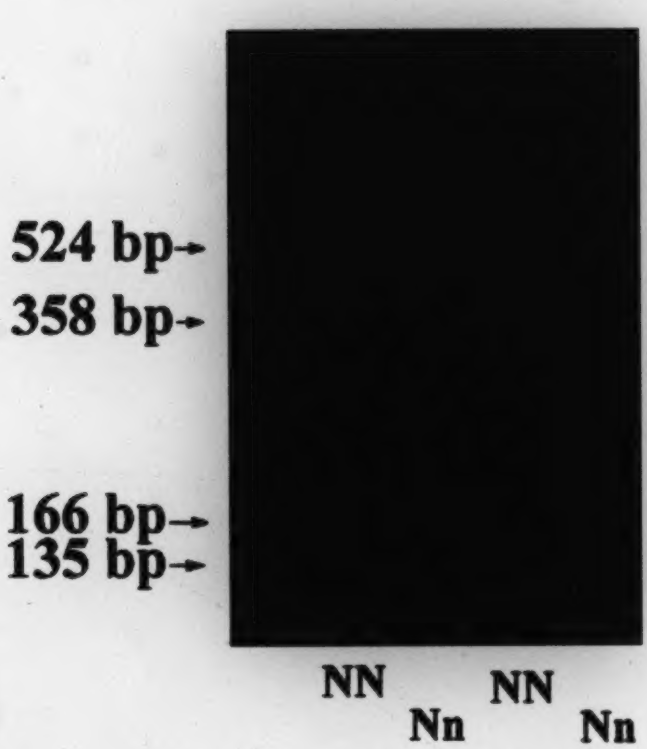


Figure 7-1. Differential BsiHKA restriction fragment patterns for wild-type and heterozygous pigs. Lanes 1 and 2 show the 524 and 135 bp bands obtained for wild-type (NN) pigs. Lanes 2 and 3 show the pattern obtained for heterozygous (Nn) pigs with additional fragments at 358 and 166 bp in addition to the 524 and 135 bp fragments.

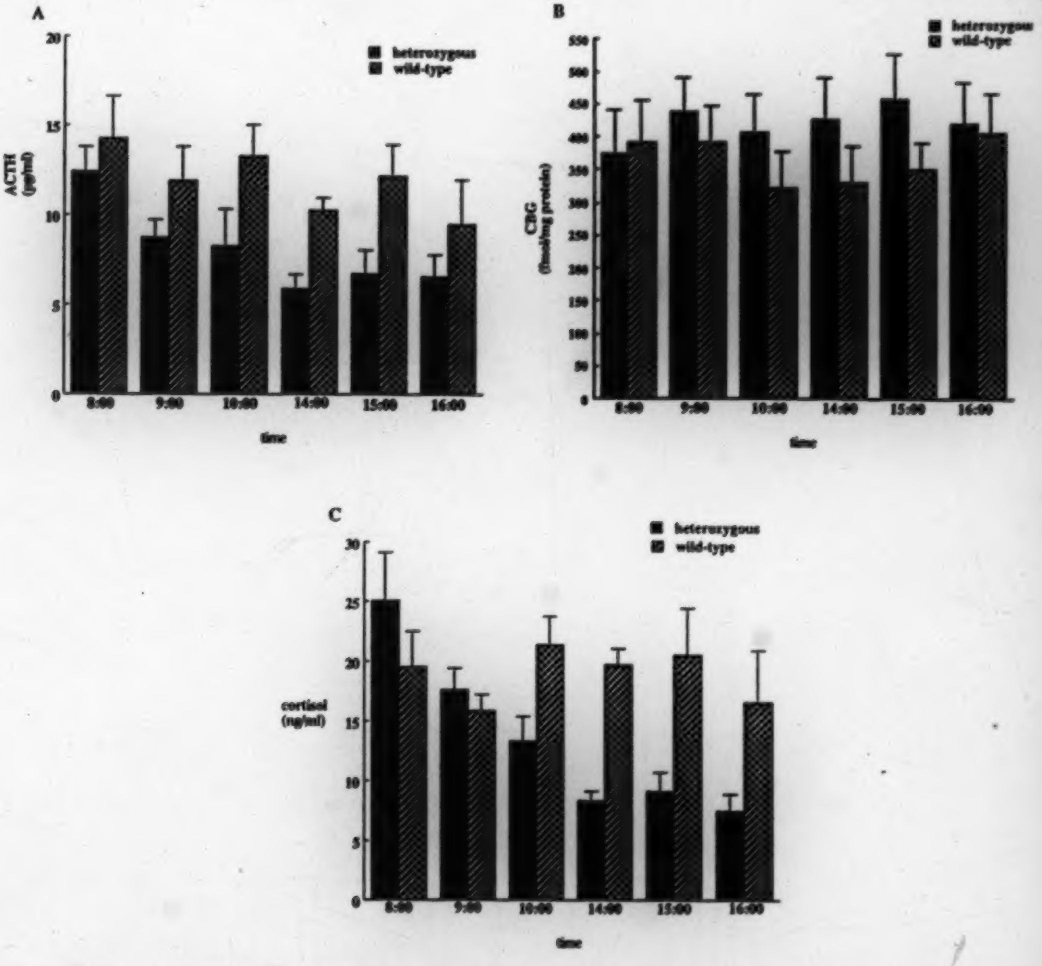


Figure 7-2. Boars heterozygous for mutated sRyR had significantly lower basal plasma cortisol and ACTH concentrations with no difference in CBC concentrations. Data for 10 heterozygous and 10 wild-type boars. A: basal plasma ACTH concentrations. B: plasma corticosteroid binding capacity (CBC). C: basal total cortisol concentrations. *significantly different at p<0.05. **significantly different at p<0.01.

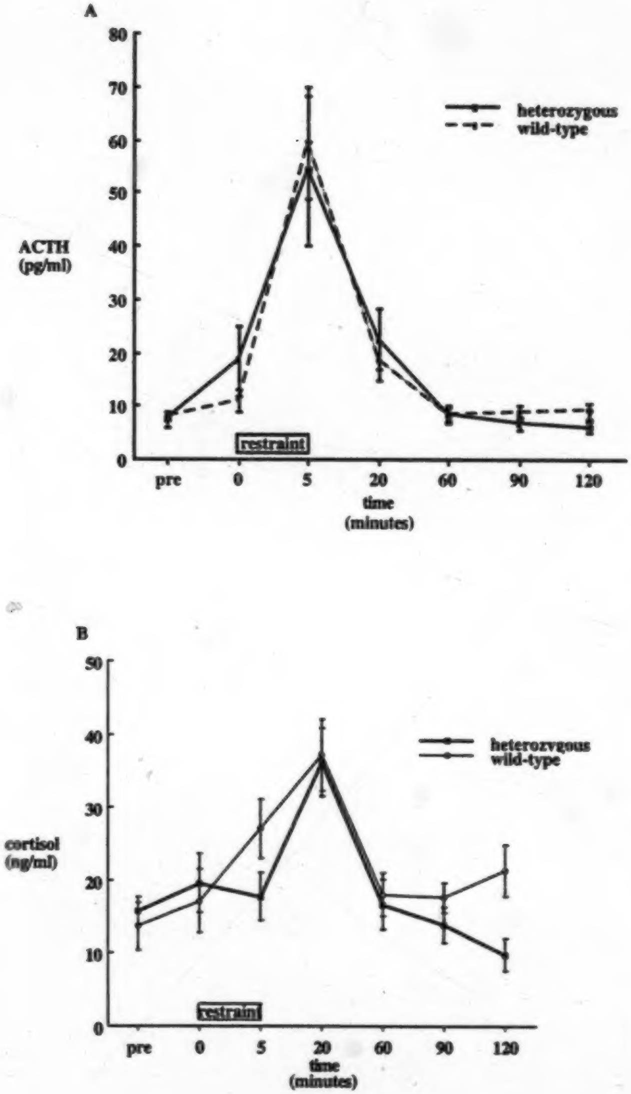
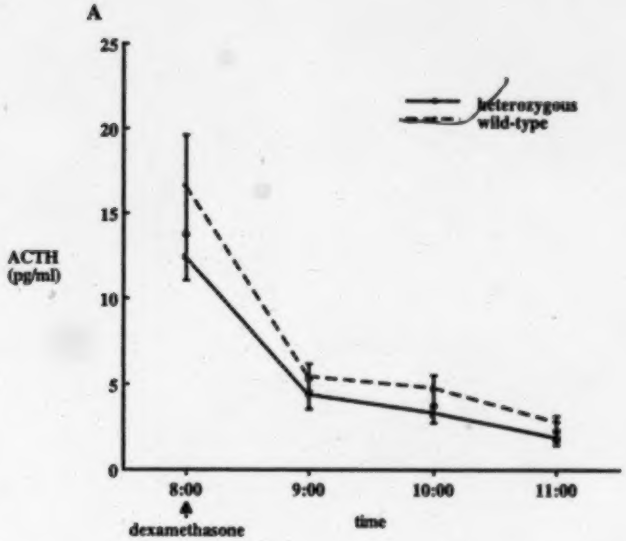


Figure 7-3. Genotype had no significant effect on ACTH or cortisol response to a 5 minute application of a nose snare. Data for 9 heterozygous and 8 wild-type pigs. A: plasma ACTH. B: plasma total cortisol. *significantly different at $p<0.05$.



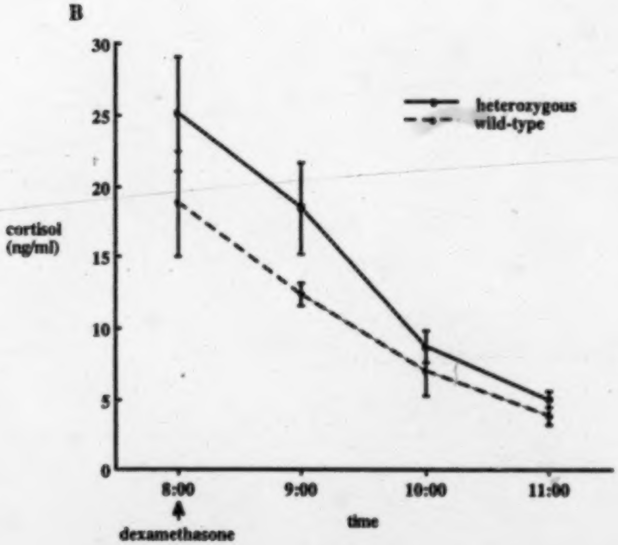


Figure 7-4. Boars heterozygous and wild-type for mutated sRyR did not significantly differ in dexamethasone suppression of ACTH and cortisol. Data for 8 heterozygous and 7 wild-type boars. 8:00 sample refers to sample obtained from basal testing. A: plasma ACTH. B: plasma total cortisol.

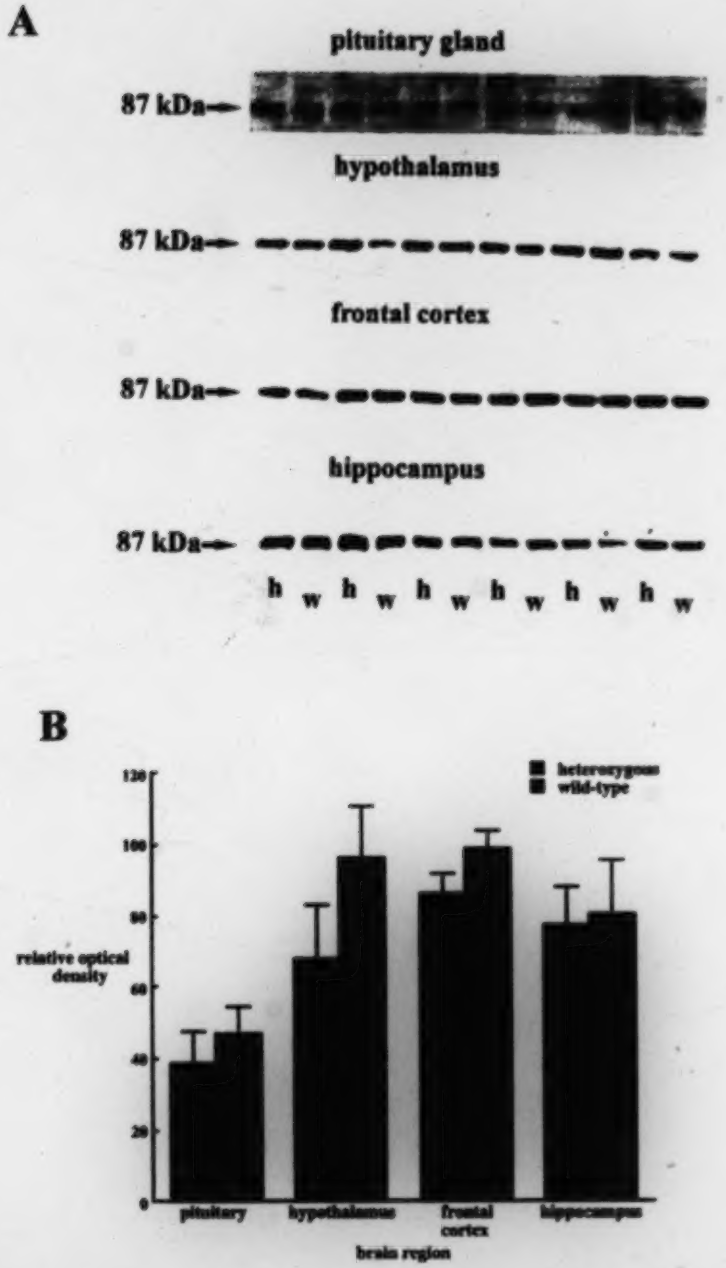
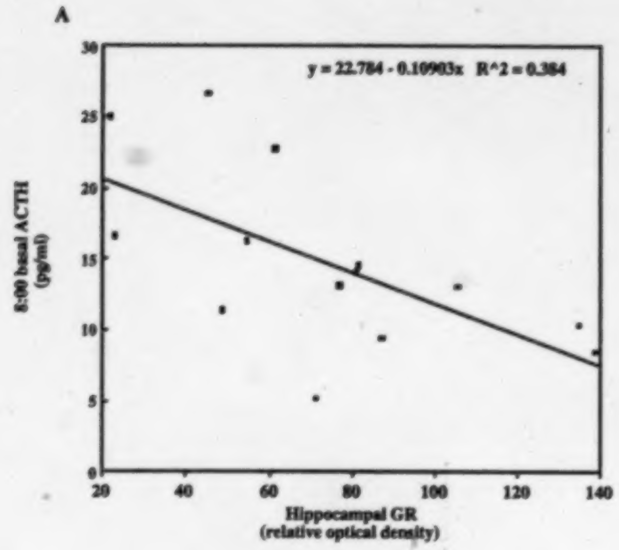


Figure 7-5. Genotype did not alter immunoreactive GR expression in brain tissue from adult pigs. A: representative western blots performed on tissue from 7 month old pigs heterozygous (h) (n=8) or wild-type (w) (n=7) for mutated sRyR. B: graphical representation of optical densitometry results.



B

Sampling time post-dexamethasone injection (minutes)	r
60	-.603*
120	-.512
180	-.142

Figure 7-6. Hippocampal GR was correlated to peak basal plasma ACTH concentrations whilst pituitary GR was correlated to dexamethasone suppressed plasma cortisol concentrations in pigs. A: significant ($p<0.05$) regression plot of immunoreactive GR and basal plasma ACTH at 8:00 across all pigs. B: correlation coefficients between immunoreactive GR and plasma cortisol concentrations during dexamethasone suppression across all pigs. *significantly different at $p<0.05$.

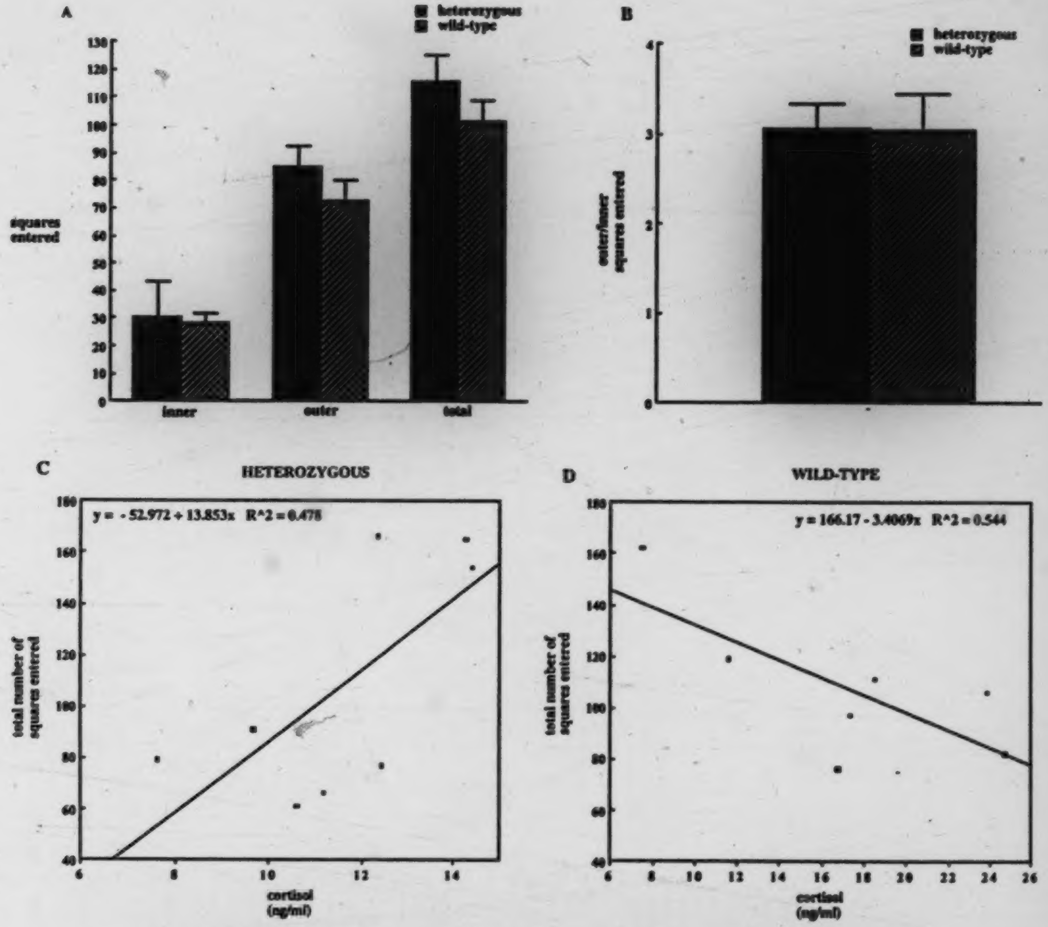


Figure 7-7. Behaviour in an open field test did not discriminate between genotypes, however, correlations with plasma cortisol concentrations during stress recovery did. Data for 10 heterozygous and 11 wild-type boars. A: number of squares entered separated into inner, outer, and the sum of both as total number of squares entered. B: ratio of the number of outer entered to inner squares entered. Significant ($p<0.05$) regressions between the total number of squares entered and plasma cortisol concentrations at 90 minutes post-snaring during separate stressor testing for heterozygous pigs (C) and wild-type pigs (D).

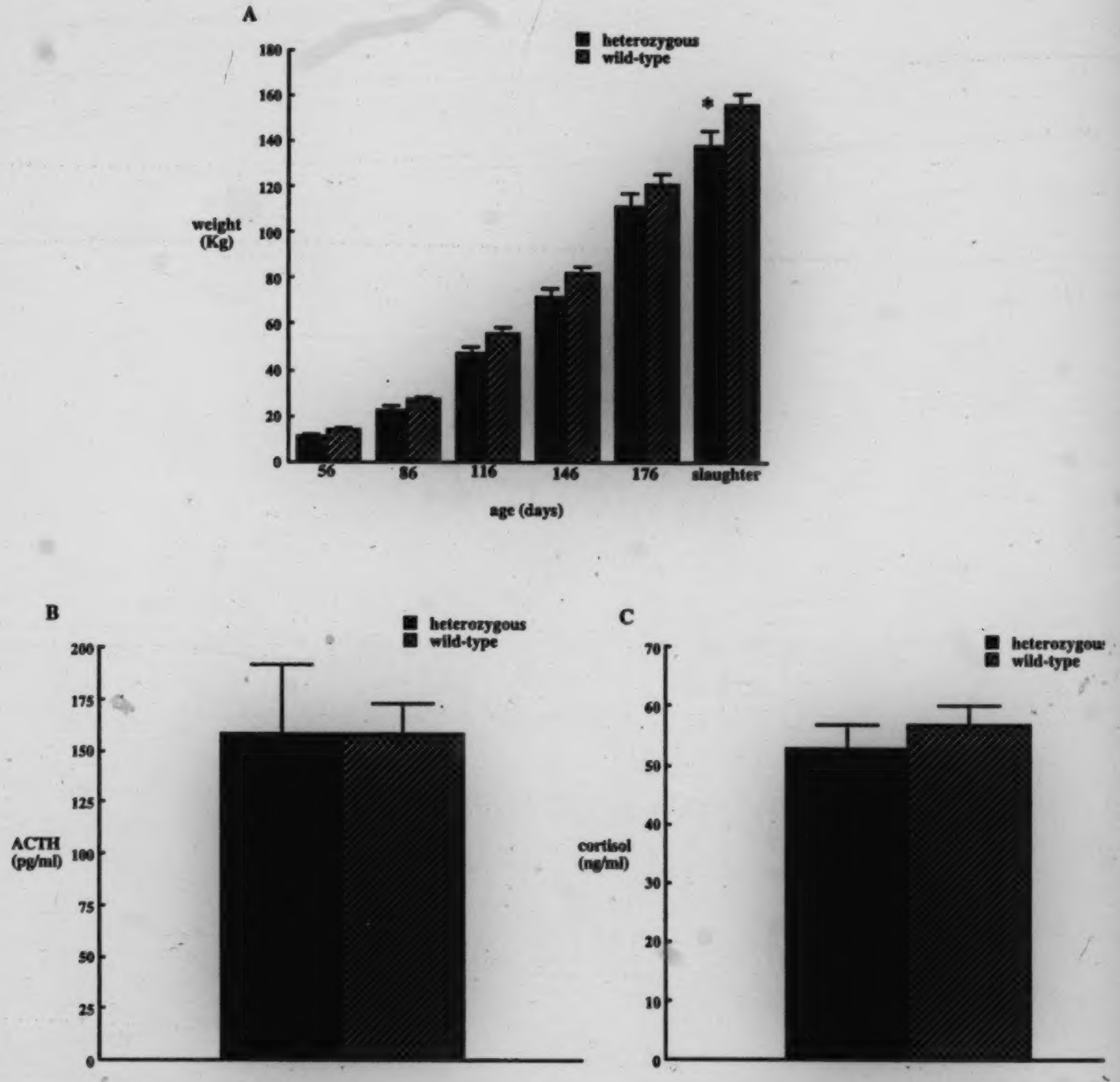


Figure 7-8. Heterozygous boars weighed less and did not differ in plasma ACTH and cortisol at slaughter. Data from 10 heterozygous and 11 wild-type boars. A: body weights obtained throughout 7 months and at the time of slaughter. B: plasma ACTH concentrations at the time of slaughter. C: plasma total cortisol concentrations at the time of slaughter. *significantly different at $p<0.05$.

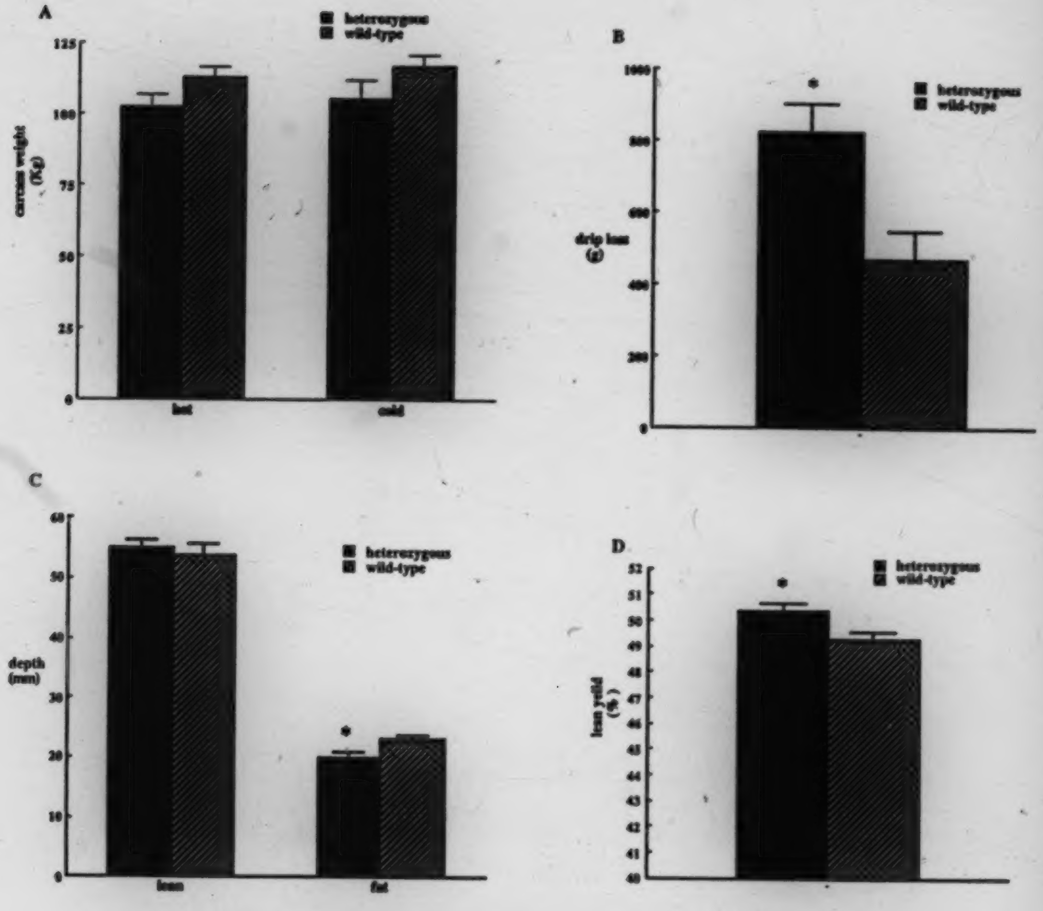


Figure 7-9. Heterozygous boars had significantly lower fat depth and higher % lean yields compared to wild-type boars. Data from 10 heterozygous and 11 wild-type boars. A: trimmed carcass weights obtained immediately after slaughter (hot) and 24 hours after slaughter (cold). B: drip loss from longissimus dorsi muscle sample measured over 48 hours. C: lean and fat depth measurements from Hennessy Probe. D: percentage lean yield obtained from lean and fat depth measurements. *significantly different at p<0.05).

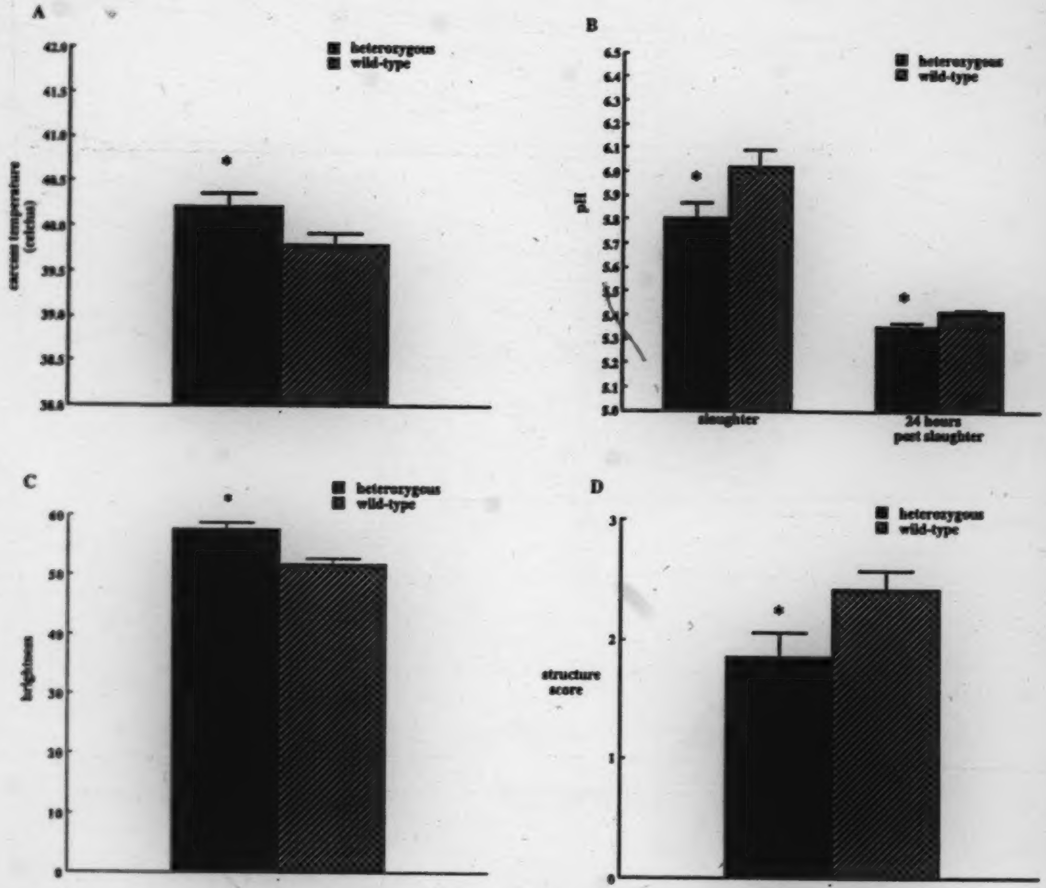


Figure 7-10. Boars heterozygous for mutated sRyR exhibited a higher incidence of PSE meat evidenced by significantly higher carcass temperature and muscle brightness scores, and significantly lower carcass pH and muscle structures scores. Data from 10 heterozygous and 11 wild-type boars. A: carcass temperature at slaughter. B: carcass pH at the time of slaughter and 24 hours later. C: objective Minolta Chroma Meter II brightness score (indicator of paleness in colour). D: subjective structure scores averaged across measures taken by 3 different graders. *significantly different at $p<0.05$).

7.5. DISCUSSION

The analysis of the effects of PSS on HPA function has revealed differences between heterozygous and wild-type pigs. The data is supported by the carcass evaluations which indicated that the heterozygous pigs did have a higher incidence of PSE meat which is indicative of PSS. For the purposes of discussion only reported studies which determined genotype via halothane or PCR based mutation detection were considered.

7.5.1. HPA function

Pigs heterozygous for the mutated sRyR had significantly lower basal ACTH and cortisol concentrations most evident during the circadian nadir (Figures 7-2A and C respectively). Both Geers et al. (1994) and Schaefer et al. (1990) have reported heterozygous pigs to have the same pre-stress or basal plasma cortisol concentrations as wild-type pigs. The cortisol values reported in these studies are equivalent to the peak stressor cortisol concentrations in this thesis and therefore, it is believed that in those reports, basal samples were not obtained. If so, our data reports the first analysis of basal cortisol concentrations in PSS heterozygous and normal pigs. Baldi et al. (1989) compared cortisol values for samples obtained either by jugular venipuncture (within 2 minutes) or anterior *vena cava* catheter and found no significant differences in cortisol concentrations. In the current work it is believed that it is not simply the effect of restraint that results in a cortisol response but it is also the effect of the presence of humans. In a survey of the literature the basal nadir cortisol concentrations described in this chapter are 3-4 fold lower than those reported by other researchers. The only other results comparable to the current data are those of Hemsworth's group (Hemsworth et al. 1987; 1986, Hemsworth and Barnett 1991) and Paterson and Pearce (1992). There is commonality between the sampling technique used in this thesis and that of Hemsworth and Paterson's work, in that remote sampling behind blinds is used, which has not been reported by other investigators. An alternate hypothesis would be that the lower cortisol concentrations described in the present work result from differences in assay techniques, pigs, or the environment. Cook et al. (1996) has measured serum cortisol concentrations using the same assay kit used for the current work, working with pigs at the Lacombe Research Centre and in that case the pre-stress (basal) samples obtained from catheterized pigs in their experiment was approximately 40% higher at the same time of sampling than those described in the present work.

Differences in basal nadir ACTH and cortisol suggest that MR receptors may differ between these two genotypes since MR rather than GR is involved in controlling basal nadir concentrations of ACTH via inhibitory signals (Bradbury et al. 1994, van Eekelen et al. 1988). Injections (100 ng i.c.v.) of RU28318 (MR antagonist) resulted in elevations of nadir corticosterone concentrations in rats, presumably by reducing inhibitory signals to the PVN (Ratka et al. 1989). Hippocampal MR has been shown to be down-regulated by maternal deprivation when examined in adults (Sutanto et al. 1996, Vazquez et al. 1996) and in these animals, tested as adults, basal nadir ACTH and corticosterone concentrations were elevated (Rots et al. 1996). Whether MR concentrations are elevated in heterozygous pigs in a structure such as the hippocampus is speculation; however ,it would be an interesting avenue of research to explore.

Stressor-induced concentrations of ACTH and cortisol did not differ between genotypes (Figures 7-3A and B respectively). It is believed that a non-remote sampling technique would induce a cortisol response equivalent to the current peak stress concentrations measured in this thesis and, therefore, obscure any differences in basal nadir plasma hormone concentrations. This might explain the discrepancy between the present work and that of Geers et al. (1994) and Schaefer et al. (1990). Roberts et al. (1996) found that the ACTH response to a blood sampling stressor (venipuncture and snaring) was significantly lower in heterozygous animals yet no differences in the cortisol response between genotypes was detected. A dysregulation of ACTH and cortisol in the present work has not been detected, as relatively low basal ACTH produced low basal cortisol and equivalent stressor-induced ACTH produced equivalent cortisol between genotypes. The stressor test in the present study represented the second time within 5 days that the pigs had been exposed to snaring (initially for catheterization) and Klemcke (1994) has demonstrated that barrows can adapt to a daily 45 minute restraint stressor with a significantly lower plasma ACTH and cortisol response when tested on day 5. The stressor used in the present work of only 5 minutes of snaring was milder and was applied a second time. However, it does confound a comparison of the differences in stress responses in the present study with that reported by Roberts et al. (1996). If the samples obtained by Schaefer et al. (1990) and Geers et al. (1994) are interpreted as stressor-induced samples (since they are equivalent to the peak cortisol concentrations achieved in the present study upon snaring), the data described in this thesis is in agreement with both of these groups since they have found no significant differences between heterozygous and wild-type pigs. The more severe ACTH and cortisol stress response elicited at the time of slaughter also did not differ between genotypes (Figures 7-8B and C), which suggests that this relationship holds true even under a novel combination of stressors. It is concluded that stressor-induced concentrations of ACTH and cortisol do not differ between pigs heterozygous for the sRyR mutation and wild-type pigs.

The two genotypes tested did not differ in negative feedback effects of cortisol on further ACTH, presumably via CRH, production during the stress response (Figure 7-3A) since there was no prolonged duration of elevated ACTH, or the suppressive effects of dexamethasone on ACTH production (Figure 7-4A). There were no differences in the cortisol response to snaring with the exception of the 120 minute sample which was obtained at 16:00 which is believed to be reflective of the relatively lower basal cortisol concentrations at this time point in heterozygous pigs. Heterozygous pigs did not differ significantly in cortisol response to dexamethasone suppression; however, they did tend to have higher cortisol level 1 hour post-injection of dexamethasone. The results showed that the plasma cortisol level at this time point was significantly correlated (r=-0.603) to pituitary GR concentrations. Dexamethasone is concentrated by the pituitary gland and not the hippocampus, unlike the situation for corticosterone, as a result of its inability to bind pituitary CBG (De Kloet et al. 1975). Therefore, perhaps the observed correlation is not unexpected. There was no effect of genotype on immunoreactive GR concentrations in this structure in spite of the trend towards a differential degree of cortisol suppression by dexamethasone. The adrenal glands tended to show a more sluggish response to the suppression of ACTH in heterozygous animals and differences between genotypes at the site of the adrenal gland may be an important factor during abrupt reductions in plasma ACTH concentrations. Evidence for this was seen in that early morning ACTH concentrations tended to be lower in heterozygous pigs, whilst cortisol concentrations tended to be higher and did not drop until 10:00 (Figures 7-2A and C).

Immunoreactive brain GR concentrations were not significantly affected by genotype (Figures 7-5A and B). A significant negative correlation was found between hippocampal GR and the peak basal plasma ACTH at 8:00 (Figure 7-6B). This is suggestive of a role of GR in the hippocampus of pigs being involved in the regulation of circadian peak ACTH concentrations and possibly negative-feedback during stress which has been demonstrated in the rat (van Eekelen 1988).

7.5.2. Behaviour

There was no significant effect of genotype on mobility scores in the open field test (Figures 7-7A and B). Correlations between the cortisol level at 90 minutes post-stressor termination did not exist across both genotypes and a correlation analysis within each genotype revealed significant correlations that differed in direction depending on the genotype (Figures 7-7C and D). Heterozygous animals showed a positive correlation between the 90 minute cortisol value compared to the negative correlation found across handled and nonhandled pigs from Chapter 6 or within the nonhandled wild-type pigs (Figure 7-7D). What is notable is that the cortisol concentrations at this time point in heterozygous animals are lower than those for the wild-type pigs. As has been suggested in Chapter 6, the relationship between cortisol and total mobility may differ depending on the absolute concentrations of cortisol. von Borrell and Ladewig (1992) found a positive correlation between adrenal response to exogenous ACTH and locomotion but their reported cortisol concentrations were much higher than those found in the present study. The positive correlation found between very low cortisol concentrations may indicate that at low basal concentrations cortisol has an activating effect on locomotion in an open field test. This might be inhibited upon increasing cortisol concentrations such as seen with a mild stressor. High concentrations of cortisol might then result in a hyper-activating effect on locomotion. A hypothesis could be developed that there exists a triphasic locomotor response to cortisol in an open field test in the absence of other important factors affecting locomotion. Undoubtably numerous other factors are likely involved in open field test locomotor behaviour. Of note however is that locomotion and the pattern of locomotion does not distinguish heterozygous from wild-type boars.

7.5.3. Weight gain and carcass measurements

Heterozygous pigs tended to have lower body weights and significance was reached at the time of slaughter (Figure 7-8A). Heterozygous pigs have been reported previously to have reduced average daily gain (Dugan et al. 1997). Carcasses from heterozygous pigs had significantly lower fat depth, which resulted in significantly higher percentage lean yield and this has also been reported previously in the literature for homozygous pigs (Dugan et al. 1997). This parallels the effects of handling in pigs on growth rate and carcass composition reported in Chapter 6, where neonatally handled pigs had significantly lower growth rates and a trend towards reduced fat depth. The common factor between the heterozygous boars and the neonatally-handled boars is the substantially lower basal nadir cortisol. The work by Akana et al.

(1985) and Devenport (1989;1992) which reported an inverted U shaped curve for body weight and corticosterone with a linear response of fat: lean with increasing corticosterone might account for this effect. It can be concluded that the lower basal nadir cortisol concentrations in heterozygous pigs may be involved in generating the lower body weights and leaner carcasses seen in this genotype.

Heterozygous pigs exhibited elevated carcass temperature at kill with lower pH both at slaughter and 24 hours later (Figures 7-10A and B respectively). Meat was of a paler colour (increases brightness: Figure 7-10C) and had a decreased structure score (Figure 7-10D). These carcass quality differences cited above are all indicative of PSE meat (Aalhus et al. 1997, Lister 1987). From this data it is concluded that the HPA differences found in heterozygous pigs are accompanied by an increased incidence of PSE meat in these animals.

7.5.4. Summary

Pigs heterozygous for the sRyR mutation known to cause PSS exhibit lower basal ACTH and cortisol with no differences in peak concentrations induced by stressors. The heterozygous animals did not differ in negative-feedback effects of cortisol on ACTH release and in fact no differences were found in central GR concentrations in any brain region examined. Open field tests were not able to discriminate between the two genotypes however heterozygous pigs, unlike wild-type pigs, showed a positive correlation between mobility and plasma cortisol which is hypothesized to represent differential activating effects of cortisol on locomotion depending on the level of cortisol. The heterozygous pigs had lower body weights and fat depths at the time of slaughter. The differences described in HPA function in this chapter were accompanied by the well-established higher incidence of PSE meat in PSS pigs. It is concluded that PSS is another source of variation in HPA function in swine.

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CHAPTER 8

BRAIN CALRETICULIN AND CALSEQUESTRIN CONCENTRATIONS IN PSS PIGS

8.1. INTRODUCTION

In the previous chapter differences have been reported in basal HPA function between pigs heterozygous and wild-type for mutated sRyR. The origin and mechanisms underlying these differences is not known. It is hypothesized that a change in some aspect of brain control of basal hormone concentrations (particularly basal nadir secretion) would be present in heterozygous animals.

Adeola et al. (1993) have reported that pigs heterozygous for the mutated sRyR which causes PSS had significantly lower hippocampal 5-HT. These data suggest that sRyR may be expressed in this brain site and that some aspect of neuronal function in the hippocampus is altered in heterozygous boars. Furuichi et al. (1994) have demonstrated the presence of sRyR throughout rabbit brain, with sRyR uniformly expressed throughout the hippocampus. If mutated sRyR were expressed in the brain the presence of "leaky" endoplasmic reticulum calcium channels may elicit compensatory alterations in the concentrations of calcium-binding proteins.

Calreticulin (CR) is the major calcium binding protein of the endoplasmic reticulum (ER) whilst calsequestrin (CS) has this function in the sarcoplasmic reticulum of skeletal and cardiac muscle and is found in association with sRyR (Treves et al. 1990). In addition, CR has been shown to impair GR binding to GR response elements in genes (Burns et al. 1994; 1997). The work described in this chapter examines the effects of PSS on these two calcium-binding proteins. We have also examined the distribution of CR and CS in selected brain sites. In addition, we have also examined whether the sRyR isoform is expressed in porcine hippocampus.

8.2. METHODS

8.2.1. Animals

All experiments were performed under the approval and guidelines of the Canadian Council on Animal Care. The pigs used for these experiments were the same as those used in Chapter 7 (n=8 heterozygous and n=7 wild-type and an additional 4 wild-type boars with brain tissue undisected). We have also used tissue from nonhandled neonatal pigs (n=6) wild-type for the sRyR mutation.

8.2.2. Euthanasia of 14 day old pigs

Pigs were injected with 40 mg/kg Ketamine HCl (Ayerst) i.m. and decapitated upon confirmation of anaesthesia. Trunk blood was collected in EDTA coated tubes (Becton Dickinson) for genotype determination. Crania were removed and the pituitary gland was collected and the frontal cortex, hypothalamus, and hippocampus were dissected out on ice within 3 minutes of decapitation and snap frozen in liquid nitrogen and stored at -72^0 C.

8.2.3. Euthanasia of adult boars

Boars were transported to an on-site meat facility at 6:00 and all animals were sacrificed by 10:00. Animals were electrically stunned and exsanguinated. Craniotomies were performed and the brains were collected along with the pituitary and snap frozen in liquid nitrogen and stored at -80^0C. Whole blood was also collected in an EDTA coated vacutainers (Becton Dickenson) and frozen at -20^0 C until analysis for genotype determination.

8.2.4. Genotype determination

Detection of the presence of mutated skeletal ryanodine receptor was performed with a modification of the procedure reported by Otsu et al. (1992). Genomic DNA was extracted from 100 µl of whole blood with 500 µl of hypotonic DNA extraction buffer (10 mM Tris and 1 mM EDTA) and centrifuged at 1,100g for 2 minutes. Proteins in the pellet were digested with 100 mg/ml proteinase K (in 50 mM KCl, 10 mM Tris, 5% (v/v) Tween 20) with incubation at 56^0C for 1 hour. Proteinase K was inactivated by boiling for 10 minutes. Samples were centrifuged at 12000 rpm for 2 minutes and the supernatant containing the DNA was used for Polymerase Chain Reaction (PCR) amplification of sequences containing the skeletal ryanodine receptor.

Forward primer sequence ($5^{\prime}$ - TCC AGT TTG CCA CAG GTC CTA CCA - $3^{\prime}$) and reverse primers ($5^{\prime}$ - ATT CAC CGG AGT GGA GTC TCT GAG - $3^{\prime}$) corresponding to intronic sequences flanking the exon of interest, were used to amplify a 659 bp sequence which contained the site of a possible thymine for cytosine substitution at bp 1843 for sRyR. This single base change creates a BsiHKA restriction site in carrier animals along with another common BsiHKA site found in both mutated and wild type DNA as a control. The PCR mix contained 1 mM of dATP, dGTP, dTTP, dCTP each (Perkin Elmer Cetus), 2 mM $MgCl_2$ (Perkin Elmer Cetus), 0.1 µM of each primer, 0.75 units of Taq DNA Polymerase (Promega), GeneAmp 10X PCR buffer II (Perkin Elmer Cetus) (final concentrations: 10 mM Tris-HCl pH 8.3, 50 mM KCl). The PCR program was 94^{0}C for 1 minute followed by 65^{0}C for 1 minute repeated for 35 cycles with a final extension time of 7 minutes at 72^{0}C. Restriction enzyme digestion was performed with 5 units of BsiHKA I (Promega) in NEB buffer (Promega) at 56^{0}C for 1 hour. Digested samples were electrophoresed on 8% (v/v) Tris-borate polyacrylamide gels and stained with ethidium bromide (0.5 µg/ml) and photographed using a Fisher Biotech polaroid camera with Polaroid Type 667 film and Wratten orange #15 filter over UV light. Carrier animals were detected by the presence of bands at 524, 358, 166, and 135 bp whilst the normal animals only showed bands at 524 and 135 bp (Chapter 7 Figure 7-1 page 231).

8.2.5. Western blotting

The hypothalamus, frontal cortex, and hippocampus was dissected from adult frozen whole brains post-freezing. Brain tissue was allowed to come to approximately -20^0 C and tissue was dissected using ice-cooled tools. Tissue samples from adult and neonatal boars were first powderized in liquid nitrogen using a pestle and mortar. An aliquots of powderized brain tissue from 15 adult boars (8 heterozygous and 7 wild-type for the mutated sRyR) and six 14 day old male pigs (wild-type) was prepared for western blotting using the detergent preparation. A 100 µl aliquot of powderized tissue was homogenized on ice, using a Vibra Cell sonicator in a 1:4 dilution of sample with ice cold TEDGM buffer (30mM Tris, 1mM EDTA, 2mM dithiothreitol, 10% glycerol (v/v), 10mM molybdate) with 1% (v/v) Triton X-100. The buffer also contained aprotinin (3.5µg/ml), AEBSF (0.4 mg/ml), leupeptin (1 µg/ml) and pepstatin (1µg/ml). The homogenates were centrifuged at 100,000g for 45 minutes at 4°C in a Beckman Ultracentrifuge. The supernatant was collected on ice and frozen at -72^{0}C. Protein concentrations were determined using the Bradford assay (Bradford 1976) with samples. 50 µg of protein per sample was added to a commercially available (Novex) 4X sample buffer (1.17 M sucrose, 563 mM tris base, 423 mM Tris-HCl, 278 mM SDS, 2.05 mM EDTA, 0.88 mM Serva Blue G250, 0.70 mM Phenol Red) with 10% (w/v) dithiothreitol added just prior to denaturing samples at 70^{0}C for 10 minutes. Samples were electrophoretically separated in a Novex minigel apparatus (Helixx

Technologies). Precast 4-12% Bis-Tris NuPAGE gels and a MOPS SDS electrophoresis buffer (1 M 3-(N-morpholino) propane sulfonic acid, 1 M tris base, 10% sodium dodecyl sulfate, 20.5 mM EDTA) were used along with 0.25% antioxidant which was purchased from Novex added to the inner chamber electrophoresis buffer. Proteins were transferred to PVDF membranes using the BioRad wet transfer apparatus with a modified version of the recommended NuPAGE transfer buffer (25 mM Tris base, 25 mM bicine, 1 mM EDTA, 0.03% (w/v) sodium dodecyl sulfate, 20% (v/v) methanol, and 0.1% (v/v) antioxidant purchased from Novex and added immediately prior to transfer). Membranes were blocked with 5% (w/v) skim milk powder. The antibodies used were generously donated by Dr. M. Michalak (Department of Biochemistry, University of Alberta) and were a goat anti-rabbit CR polyclonal and a rabbit anti-dog cardiac calsequestrin. Antibodies were used at a dilution of 1:4000 and 0.5% porcine plasma was included with the CR antibody to inhibit nonspecific binding to albumin. The horseradish peroxidase-linked secondary used with the CR antibody was an anti-goat IgG purchased from Jackson Immunochemicals (Westgrove, Pa.). A 1: 4000 dilution of the primary antibodies in TBS-T (20 mM Tris base, 140 mM NaCl and 0.01% (v/v) Tween 20) containing 0.5% (w/v) skim milk powder(0.5% (v/v) porcine plasma with the CR antibody) was incubated with the membrane overnight at room temperature. The membranes were washed with TBST after which a 1: 5000 dilution of the horseradish peroxidase linked secondary antibody was incubated with the membrane for 1 hour at room temperature. Hyperfilms (Amersham, Toronto, Ont. CA.) were exposed to chemiluminescence via a horseradish peroxidase linked-secondary antibody and the ECL detection system (Amersham, Toronto, Ont. CA.). The intensity of bands were analyzed using relative optical densities determined using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catharines, Ontario). A polyclonal (rabbit) anti-chicken tubulin antibody was applied to membranes and detected to ensure equivalent protein loading. All western blots were performed in at least duplicate.

8.2.6. *In situ* hybridization

For *in situ* hybridization of sRyR mRNA, all solutions were prepared in sterile water treated with 0.1% diethylpyrocarbonate. Serial 15 µm thick coronal sections were cut using a Microm HM 500 M cryostat through the dorsal hippocampus (AP +8.0 (Salinas-Zeballos et al. 1986)) from 4 wild-type adult boars (euthanized as described but tissue not processed for western blotting) at -15^{0}C and thaw-mounted onto 0.01% poly-L-lysine-coated slides. Sections were desiccated overnight at 5^{0}C and then stored at -80^{0}C until use. pRR229 plasmid which contained the rabbit RyR skeletal isoform specific (Takeshima et al. 1989) sequence was kindly donated by Dr. H. Takeshima (Departments of Medical Chemistry and Molecular Genetics, Kyoto University). The plasmid was linearized using Bgl II and a 412 bp antisense probe was synthesized using T_3 polymerase (T_7 was used to generate sense probes) and 20 µM of 800 Ci/mmol [^{35}S]UTP. The 412 bp fragment shared 89% homology with porcine sRyR. Sections were brought to room temperature (10 minutes) and then post-fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 minutes followed by one 5 minute wash in 2X SSC (0.3M sodium chloride, 0.03 M sodium citrate). Sections were then treated with 0.25% (v/v) acetic anhydride added fresh to 1.4% (v/v) triethanolamine and 0.3% (v/v) hydrochloric acid for 10 minutes. Slides were rinsed in 2X SSC followed by progressive dehydration in 50%, 70%, 95%, and 100% (v/v) ethanol prior to delipidation in chloroform for 10 minutes. Sections were then partially rehydrated in 100% followed by 95%(v/v) ethanol. Antisense or sense probes were heat denatured at 65^{0}C and added to hybridization buffer (50% (v/v) deionized formamide, 10 mM dithiothreitol, 10 mM Tris (pH 7.5), 600 mM sodium chloride, 1 mM EDTA, 10% (w/v) dextran sulphate, 1X Denhardt's solution, 100 µg/ml salmon sperm DNA, 100 µg/ml yeast tRNA) to a final concentration of 14 x 10^6 cpm/ml. Hybridization mix (900 µl) was added to each section (1 x10^6 cpm/section) and hybridized for 18 hours at 55^{0}C in sealed humid chambers. Following hybridization, slides were rinsed twice in 2X SSC and treated with RNase A (20 µg/ml RNase A, 500 mM sodium chloride, 10 mM Tris, 1 mM EDTA) for 30 minutes at room temperature. Slides were then washed twice for 10 minutes in 2X SSC at room temperature and a single 1 hour wash in 0.5X SSC containing 0.05% formamide at 37^0 C. Sections were then progressively dehydrated using 50%, 70%, and 90% (v/v) ethanol in 0.3 M ammonium acetate and air dried and exposed to Bmax film (Amersham, Toronto, Ont. CA.) to ensure the presence of specific labelling. Slides were then dipped in photographic emulsion (Kodak NTB-2) and exposed at 4^0C for 30 days before being developed and counterstained with Cresyl Violet. The hybridization signal within dorsal hippocampal sub-regions was quantified by grain counting over individual hippocampal neurons using a light microscope under brightfield illumination. For each cell field, grains over 40 individual neurons/section were counted and counting was performed on 4 sections per animal. Following subtraction of background, mean values were derived for each hippocampal cell field for each animal. Background ranged between 10 and 15% of values found over hippocampal cells.

8.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the Super Anova Statsview software package from Abacus Concepts. Data presentation is in the form of means with standard errors of the means. Group data for *In situ* hybridization and western blotting results were analyzed using unpaired Students t tests. Statistical analyses of brain region differences in expression was performed using 1 factor ANOVA with brain region as the factor followed by Student-Newman-Keuls *post hoc* tests.

8.4. RESULTS

CR expression was significantly ($p<0.0001$) higher in the pituitary gland compared to the hypothalamus, frontal cortex, and hippocampus which did not differ from each other in CR concentrations in both neonatal and adult pigs (Figures 8-1A, B and 8-2A, B respectively). Neonatal pigs had significantly ($p<0.05$) higher concentrations of pituitary CR than adults, with no obvious developmental effects on CR in neural tissue (Figures 8-3A and B). Immunoreactive calsequestrin was not detected in the pituitary gland and was barely detectable in the hypothalamus and frontal cortex. Significantly ($p<0.0001$) higher concentrations of CS expression were seen in the hippocampus (Figures 8-4A and B).

Immunoreactive CR was not affected by genotype in any brain region examined (Figures 8-5A and B) in adult boars. Immunoreactive CS was only analyzed in the hippocampus due to the barely detectable concentrations in the hypothalamus and frontal cortex. Boars heterozygous for the sRyR mutation had significantly ($p<0.0018$) lower concentrations of immunoreactive CS in the hippocampus (Figures 8-6A and B).

In situ hybridization with the sRyR probe revealed that mRNA for this protein was indeed present in the porcine hippocampus and was evenly distributed throughout all cell subfields (Figures 8-7A and B).

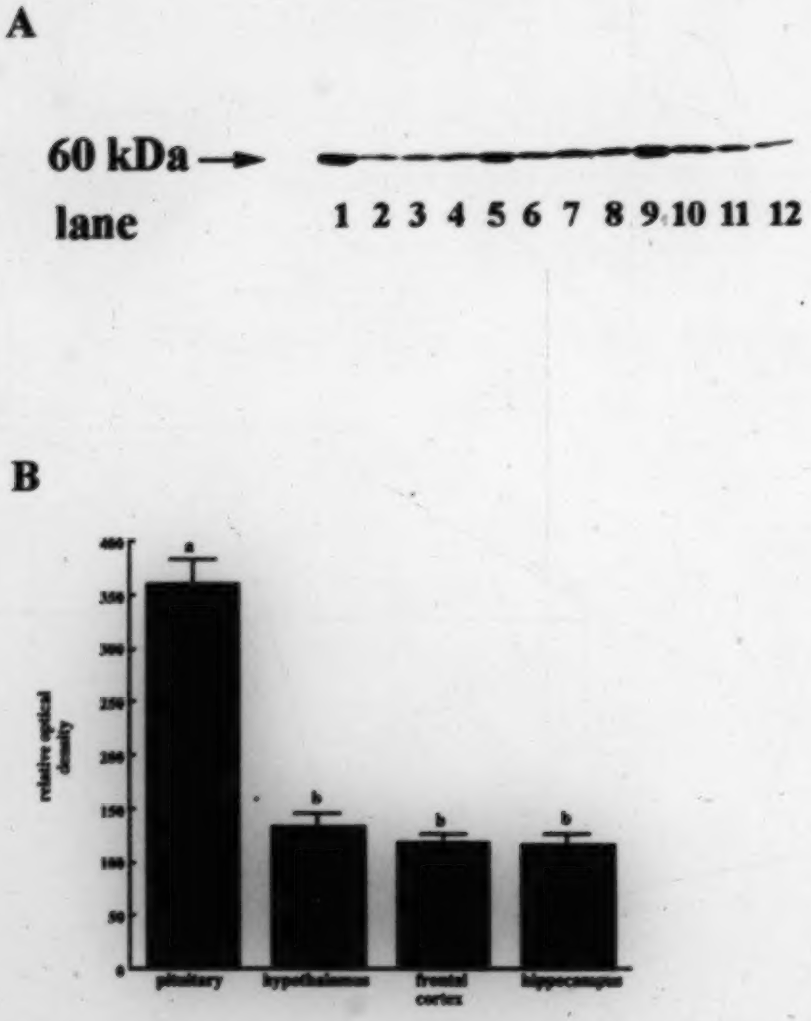

Figure 8-1. Regional distribution of CR in neonatal porcine brain tissue. Panel A: representative western blot using three pigs (total n=6). Sample identity is pituitary gland (lanes 1, 5, and 9), hypothalamus (lanes 2, 6, and 10), frontal cortex (lanes 3, 7, and 11), and hippocampus (lanes 4, 8, and 12). Panel B: graphical representation of relative optical densities. Sites with different letters differ significantly ($p<0.0001$).

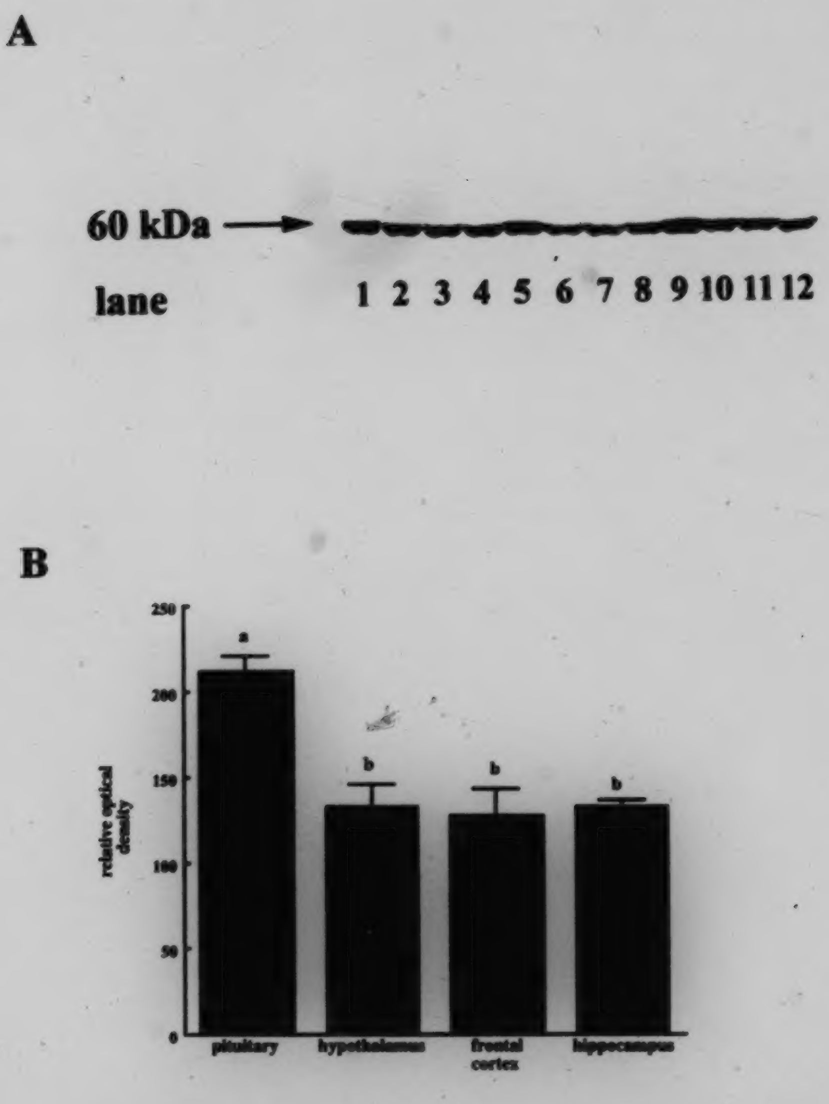

Figure 8-2. Regional distribution of CR in brain tissue from adult boars. Panel A: representative western blot using three pigs (total n=6). Sample identity is pituitary gland (lanes 1, 5, and 9), hypothalamus (lanes 2, 6, and 10), frontal cortex (lanes 3, 7, and 11), and hippocampus (lanes 4, 8, and 12). Panel B: graphical representation of relative optical densities. Sites with different letters differ significantly ($p<0.0001$).

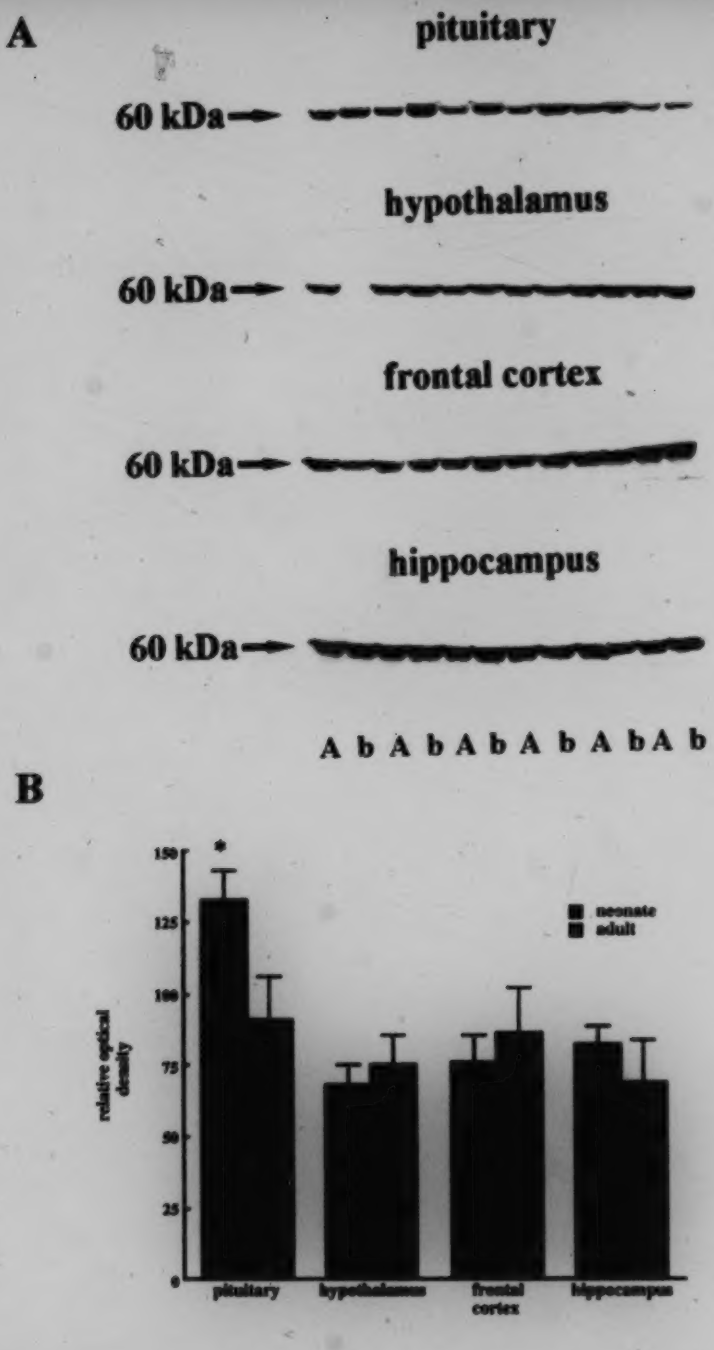

Figure 8-3. Regional distribution of CR in adult and neonatal porcine brain tissue. Panel A: representative western blots of 6 14 day old male pigs and 6 7 month old boars. Lanes labelled with b are neonatal tissue and A labelled lanes are adult tissue. Panel B: graphical representation of optical densities. Sites with different letters differ significantly (p<0.05).

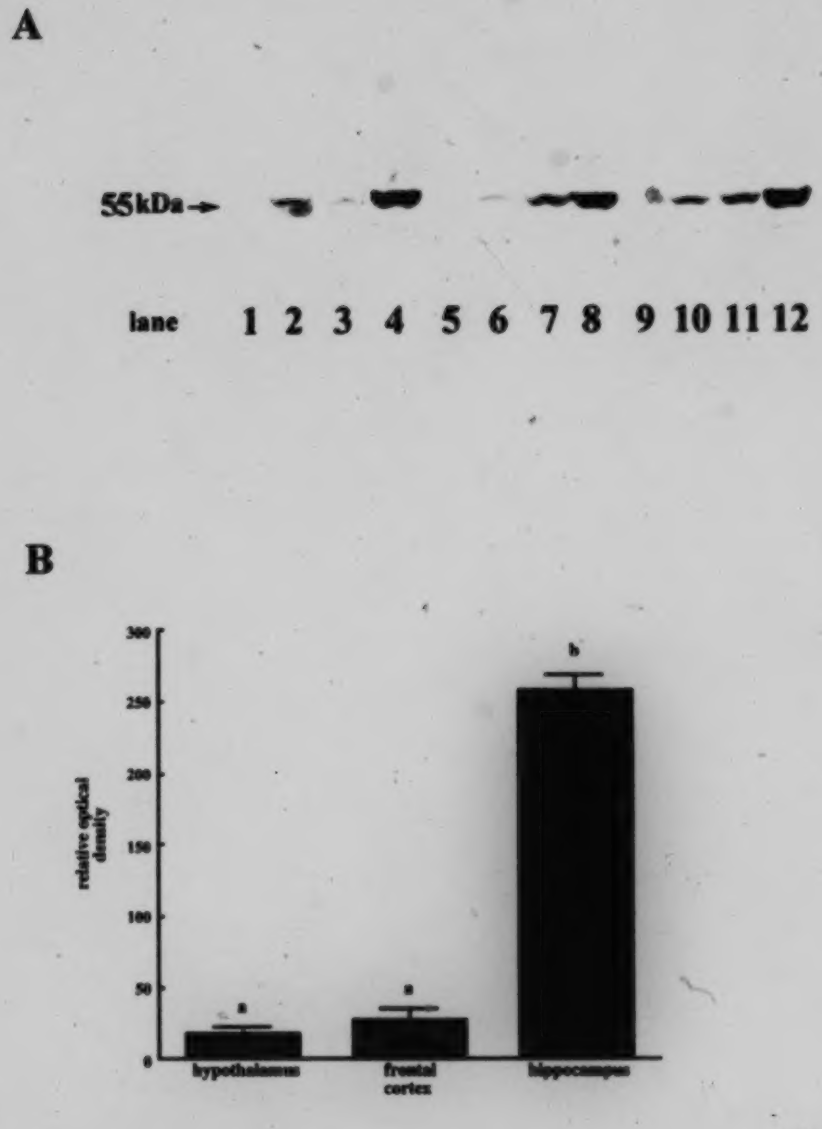

Figure 8-4. Regional distribution of CS in brain tissue from adult boars. Panel A: representative western blot using three pigs (total n=6). Sample identity is pituitary gland (lanes 1, 5, and 9), hypothalamus (lanes 2, 6, and 10), frontal cortex (lanes 3, 7, and 11), and hippocampus (lanes 4, 8, and 12). Panel B: graphical representation of relative optical densities. Sites with different letters differ significantly ($p<0.0001$).

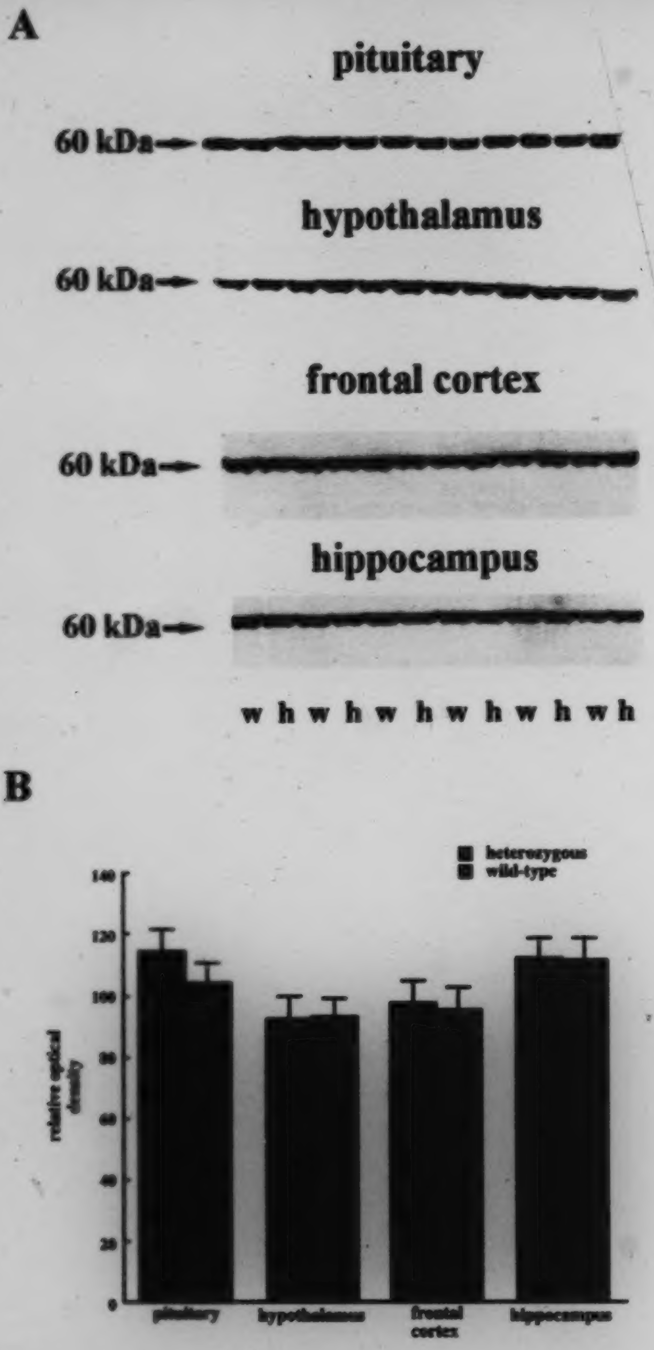

Figure 8-5. Boars heterozygous for mutated sRyR did not differ in immunoreactive CR expression in any brain region examined. A: representative western blots performed on tissue from 7 month old pigs heterozygous (h) (n=8) or wild-type (w) (n=7) for mutated sRyR. B: graphical representation of optical densitometry results.

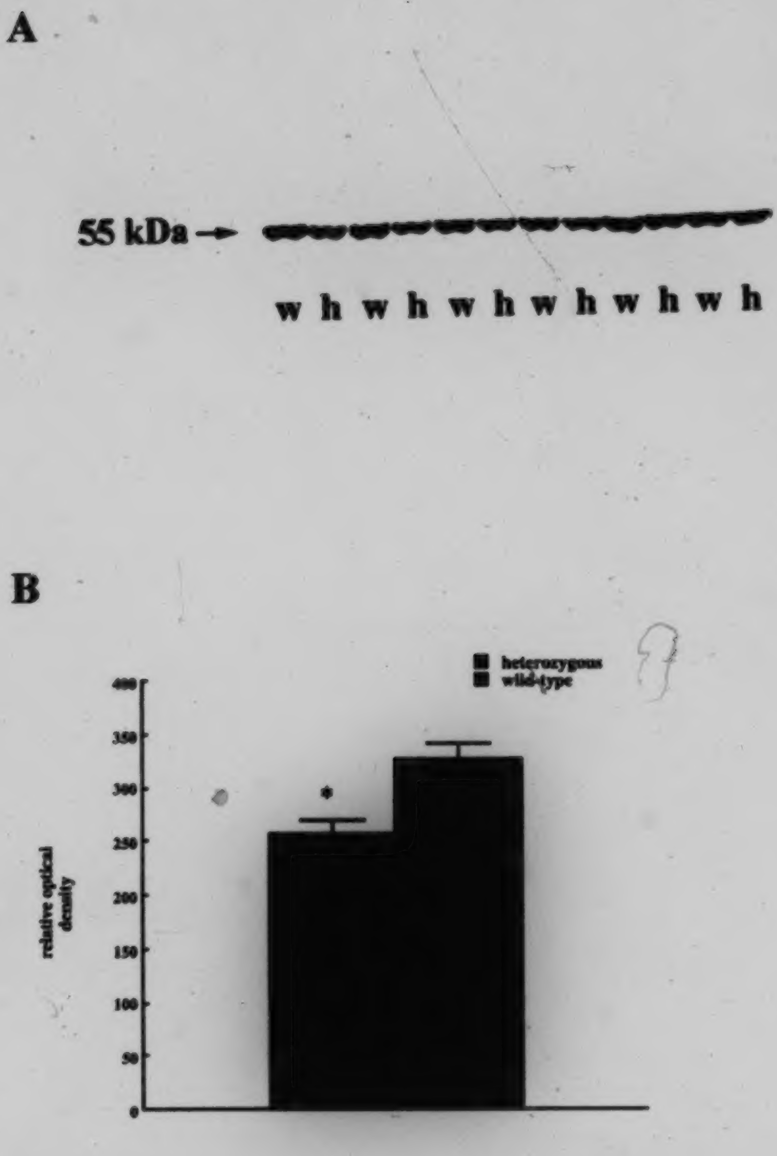

Figure 8-6. Boars heterozygous for mutated sRyR had significantly lower immunoreactive CS expression in the hippocampus. A: representative western blots performed on tissue from 7 month old pigs heterozygous (h) (n=8) or wild-type (w) (n=7) for mutated sRyR. B: graphical representation of optical densitometry results. *significantly different at $p<0.0018$.

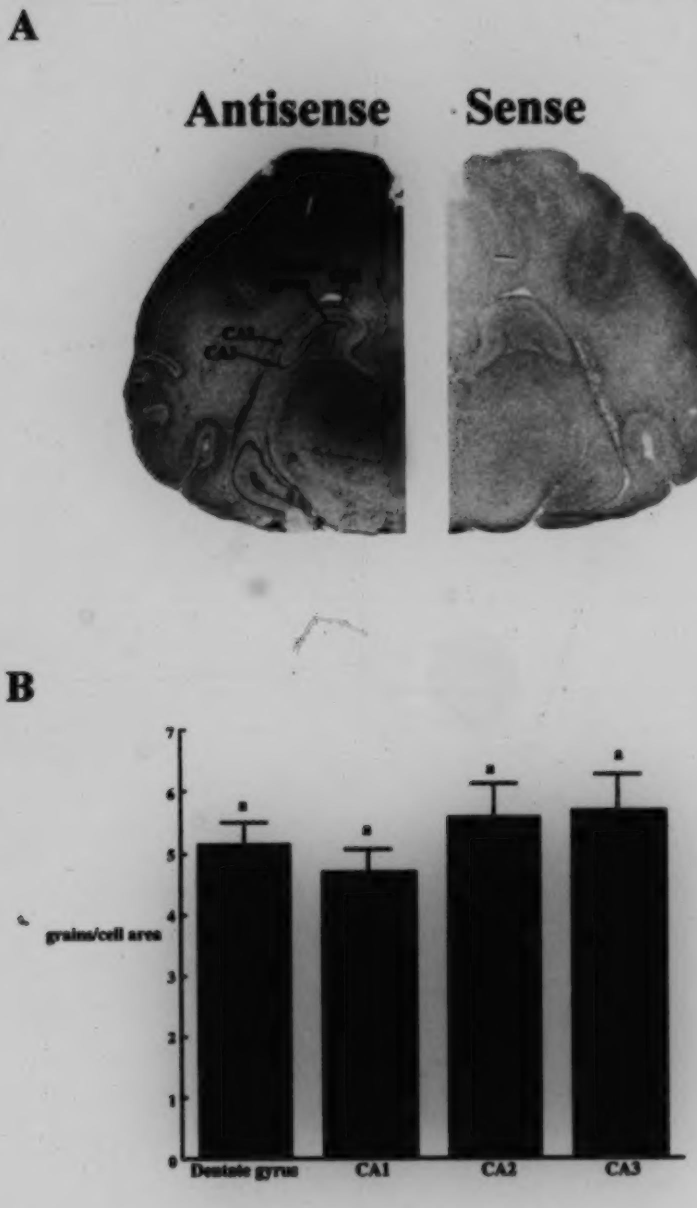

Figure 8-7. mRNA for the skeletal isoform of the ryanodine receptor was expressed in the porcine hippocampus. A: representative antisense and sense hybridization of ^{35}S labelled sRyR riboprobe from 4 wild-type pigs. B: graphical representation of grains per cell area across hippocampal cell subfields. Means with the same letter do not differ significantly.

8.5. DISCUSSION

Calreticulin has been shown to be expressed in whole brain microsomal fractions prepared from rabbits (Treves et al. 1990) and frogs (Treves et al. 1991). The current data is believed to represent the first analysis of CR expression in discrete brain regions. Additionally, this is the first report of calreticulin expression in porcine tissue. Calreticulin binds Ca^{2+} with high affinity and is proposed to be one of the major Ca^{2+} storage proteins of the ER in non-muscle cells (Milner et al. 1991, Baksh and Michalak 1991, Mery et al. 1996). CR has also been proposed to function as a stress protein (Guan et al. 1991), a molecular chaperone (Guan et al. 1991), an α-integrin-binding protein (Leung-Hagesteijn et al. 1994), and to regulate the DNA-binding ability of steroid receptors (Dedhar et al. 1994, Burns et al. 1994; 1997). The function of CR in brain tissue is not known, however, it could be involved in regulating Ca^{2+} mediated signalling, steroid receptor function, or anchoring of synapses to the extracelluar matrix during development. The demonstration of high concentrations in the porcine pituitary gland (non-neural) may indicate important functional roles in this structure. In addition, the relatively higher concentrations in neonates versus adults indicates a possible role in development. The stress or slaughter would be greater in the adults and therefore they may show higher levels of CR in the nucleus which occurs upon dexamethasone treatment of LM(TK$^-$) cells which would confound results. The difference between neonatal and adult concentrations of CR must be approached with caution however, since these differences are detected against a background of changes in other proteins.

Calsequestrin functions as the major calcium sequestering protein of skeletal muscle tissue (Lytton and MacLennan 1991) and, although no cardiac calsequestrin has been detected in rabbit whole brain microsomal preparations (Treves et al. 1990), it has been shown to be present in Purkinje neurons of the cerebellum of embryonic chickens (Sacchetto et al. 1995). The data presented in this chapter represents the first report of the presence of cardiac calsequestrin in neural tissue in pigs. Of interest is the relatively high concentrations of CS detected in the hippocampus compared to other brain regions which may imply site-specific differentiation of the functional role of CS. Also of note was the absence of CS in the pituitary gland which had the highest expression of CR. This may indicate that high concentrations of CR render the requirement of CS redundant if in fact the roles of CR and CS are interchangeable. In addition, the pituitary is a non-neural tissue and CS has been shown to be absent in non-skeletal muscle tissue such as liver and smooth muscle and is largely confined to skeletal and cardiac muscle with very small amounts in uterine smooth muscle (Treves et al. 1991, Milner et al. 1991). The lack of previous detection of CS in whole brain preparations is possibly due to the fact that discrete areas of enriched expression (such as the hippocampus) would be diluted out by relatively low or non-existent expression in other regions of the brain. In addition, porcine tissue has not previously been examined and, therefore, the expression of CS in this study may reflect a species difference.

The presence of mutated sRyR did not result in differences in CR expression in any brain region examined (Figures 8-5A and B). Calreticulin (mRNA and protein) expression has been shown to be upregulated by calcium ionophores or by the pharmacological depletion of ER calcium stores in a manner independent of extraluminal calcium content (Llewellyn et al. 1995; 1996, Waser et al. 1997, Nguyen et al. 1996). Heterozygous muscle bundles have contractile responses similar to wild-type muscle in the absence of triggering agents (ie. halothane) (Gallant et al. 1989). Calcium release properties of sRyR channels isolated from heterozygous muscle tissue shows "percent time open" values intermediate to homozygous and wild-type channels possibly resulting from the different possible combinations of mutant and wild-type subunits in forming the homotetramer (Shomer et al. 1995). The absence of differences in CR concentrations *in vivo* reported in the current work may be due to a lesser degree of calcium efflux from the ER compared to those induced by pharmacological treatments. If near complete depletion were required prior to upregulation of CR it is not unexpected that upregulation in brain tissue from these pigs was not seen.

Calsequestrin concentrations were significantly lower in hippocampal tissue from heterozygous pigs compared to wild-type pigs (Figures 8-6A and

B). This at first seemed paradoxical in light of the reported effects of calcium depletion on calreticulin. In muscle cells CS is the principal calcium-binding protein of the SR and is proposed to bind calcium and reduce the Ca^{2+} gradient against which the Ca^{2+}-ATPase pump works to refill SR calcium stores (Lytton and MacLennan 1991). Sarcolemmal or transverse tubule membranes from homozygous pigs were found to accumulate 25% less calcium compared to those from normal pigs after 2 or 20 minutes of calcium uptake (Ervasti et al. 1989, Mickelson et al. 1987) which might relate to decreased CS. Calsequestrin has been found to be associated with RyR channels (Yano and Zarain-Herzberg 1994) and it may be possible that the presence of mutated sRyR subunits may decrease this association and result in reduced concentrations of CS. In addition, crude membrane preparations from homozygous pigs contain less [^{3}H]ryanodine binding capacity (no change in affinity) (Mickelson and Louis 1996) and it is possible that CS concentrations are downregulated in response to decreased sRyR. Binding of calcium to CS renders CS more resistant to protease digestion due to conformational changes (Mitchell et al. 1988); therefore, if ER calcium stores were lower increased CS degradation may result. All of the above are speculative as to the origin of the reductions in CS observed in heterozygous pigs which remains to be determined.

In light of the changes in hippocampal CS that have been observed (Figure 8-6) and the significantly lower 5-HT content in the hippocampus of heterozygous pigs reported by Adeola et al. 1993, *in situ* hybridization was used with a sRyR specific riboprobe in order to determine if mRNA for this isoform of RyR was expressed in the hippocampus. sRyR was expressed in the porcine hippocampus and its expression was equivalent across all cell subfields as has also been reported for the rabbit hippocampus (medium to high concentrations of expression) (Furuichi et al. 1994). Furuichi et al. (1994) have reported that sRyR concentrations are low in the hypothalamus and low to medium in the frontal cortex in rabbits. Sundaresan et al. (1997) have reported no expression of sRyR in rat pituitaries using *in situ* hybridization. If this distribution pattern were the same in pigs it would be consistent with a lack of CS expression in pituitary gland and the low level of expression in the hypothalamus and frontal cortex observed in the current study. The present data indicates that sRyR mRNA is expressed in porcine hippocampus; however, its impact on neuronal function is not known.

Summary

The presence of immunoreactive CR and CS in porcine brain tissue has been demonstrated. High concentrations of CR expression in the pituitary gland were found while and concentrations of expression in the hypothalamus, frontal cortex, and hippocampus were equivalent. The distribution of CS was dramatically different from the CR results, in that no immunoreactive CS was detected in the pituitary gland and only very faint signals were found in the hypothalamus and frontal cortex. Significantly higher concentrations were observed in the hippocampus. The presence of mutated sRyR in heterozygous pigs had no detectable effect on CR concentrations in any brain region examined, however, hippocampal CS was significantly down-regulated. In support of this change we have demonstrated the presence of sRyR mRNA in the hippocampus as well. The relationship and functional significance of reductions in hippocampal CS and the presence of mutated sRyR in this structure remain to be elucidated.

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CHAPTER 9

GENERAL DISCUSSION

Our lives reflect a continuum of coping with a variety of stressors, whereupon the success with which we respond (or limit our response) can determine aspects of our physiologic success. In the commercial production, pigs must through a myriad of stressors throughout the production cycle. The success with which an animal is able to respond, or limit its response, to these stressors will ultimately determine the financial success of such production. It would, of course, be beneficial to eliminate or at least minimize stressors in barns. However, this is not practical and an alternative objective might be to ensure that production herds are composed of animals which exhibit "appropriate" responses to stressors, such that stress-induced impairment in their performance is minimized. Addressing this objective would necessitate a clear understanding of individual differences in HPA function, the basis for these differences. One of the factors which controls the degree of responsivity of an animal to stressors is that of individual differences of a genetic or experiential origin (Vogel 1985). The objectives of this thesis were to examine sources of individual differences in HPA function in swine. These objectives have been addressed by examining the central glucocorticoid receptor and its role in stress HPA function, and the impact of the early neonatal environment on central GR and HPA function. In addition, the HPA function has been examined in PSS pigs which have been proposed to differ in HPA function due to genetic differences.

In Chapters 2 and 3 of this thesis issues relevant to the accurate measurement of central GR in pigs have been addressed, and appropriate methodologies validated for the rest of the thesis. Chapters 4 and 5 describe the role of increased 5-HT during early life in pigs and rats and its relationship to HPA function. In Chapter 6 the rat neonatal handling paradigm (proposed mechanism of action via 5-HT) was applied to pigs and HPA function was examined in adults. Chapters 7 and 8 dealt with examinations of the influence of mutated sRyR on HPA function and central calcium-binding proteins as proposed mechanisms for altered HPA function. Certain unifying themes were present across experiments and these include the roles of central GR and MR, the importance of remote sampling, the relationship between basal HPA function and body weight, and the incidence of adrenal insensitivity.

Chapters 2 and 3

In order to describe HPA function in pigs an attempt was made to provide information on the hypothalamic-pituitary aspects of this axis as well as higher brain structures (frontal cortex and hippocampus). Central GRs have been shown to be involved in controlling stress responses and peak circadian HPA function (de Kloet 1991, Meaney et al. 1993) and, therefore, our attention was focused on this receptor in the brain. Analyzing steroid receptors is difficult due to nuclear localizations, and in the case of GR, discrete populations of this receptor are found partitioned into the nucleus and cytoplasm (Pratt et al. 1989). Traditional receptor binding assays do not measure receptors which are in a conformation that is unable to bind ligand. To circumvent this problem adrenalectomy 12-16 hours prior to sacrifice has been used by other investigators to prevent activation of receptors by endogenous ligands (McEwen et al. 1974). To avoid this problem in the present work western blotting with antibodies demonstrated to bind to all forms of the GR have been used. In Chapter 2 problems concerning the lack of specificity of one of the commercial antibodies (#57) against GR were revealed. This lack of specificity was seen in that a doublet was obtained with an upper band that does not correspond to a ß-isoform of the receptor. This upper band may represent a pro-form of the receptor which upon post-translational modifications (to date unknown) is altered into the smaller functional GR. In contrast, the lower band found in pigs binds the ligand dexamethasone. Since the identity of the higher molecular weight band was not known, another antibody (#51 GR antibody) was used for subsequent experiments since it performed consistently and only recognized the specific receptor in the absence of nonspecific bands. In fact, use of this antibody also demonstrated an increase in signal intensity in hippocampal tissue from long-term ADX rats: a phenomenon known to occur (ODonnell et al. 1995).

The effect of tissue preparation technique on the isolation of GR populations and the quantification of regional expression as well as developmental changes in GR expression was an important issue which was addressed in Chapter 3. The advent of the NU-PAGE gel system allowed the use of whole cell extract preparations which use relatively high concentrations of salt to disrupt nuclear membranes and binding of GR to chromatin. Such conditions were incompatible with previously used gel electrophoresis. It was found that regional distribution and developmental changes in GR were confounded by the presence of other proteins in the high salt preparations. This WCE technique increased the liberation of many other proteins in addition to GR and it has been hypothesized that this effectively dilutes out the percentage of GR as a percent of the total protein population. In regional distribution studies the comparison of non-neural tissue (pituitary gland) to neural tissue proved to be difficult as protein dilution effects in the pituitary were most dramatic. Regardless of the preparation technique used it was found that central distribution of GR in the pig was unique, in that in no case did it parallel what had been found in the rat. In the pig, it was demonstrated that cortical structures contained the highest concentrations of GR followed by the hippocampus and hypothalamus using the WCE preparation. With respect to development it was shown that a detergent preparation method for isolating GRs resulted in identical developmental patterns in the pig compared to that found in the rat (results of receptor binding assays with soluble cytosolic preparations) in that GR concentrations in neural tissues were increased in adult pigs compared to neonatal pigs whereas is the pituitary the GR concentrations tended to be decreased in adult pigs. The WCE preparation, in contrast, only showed an effect of developmental age in the hypothalamus. This was hypothesized to be due to the diluting effects on GR abundance by other proteins which are undergoing developmental upregulation. The conclusions from this part of the work are that regional or developmental effects on GR as revealed by western blotting will be confounded by changes in other proteins.

Chapters 4 and 5

After establishing suitable methodologies for measuring central GR in pigs, as reflected by the significant correlations to dexamethasone suppression and peak ACTH basal levels, a second set of experiments was carried out to analyze the effects of augmented 5-HT on HPA function. The effects of quipazine administration neonatally in rats was assessed since the neonatal handling effect was developed in this species and 5-HT has been shown to be involved in these effects in rats (Meaney et al. 1992, Mitchell et al. 1990). From this work it was demonstrated that a stress hyporesponsive period does not occur in the rat since effective GR signalling (activation and translocation) occurs in the neonate at concentrations equivalent to those found in the adult rat. The current work provides support to that previously described by Viau et al. (1996) using receptor binding assays. In addition, Walker et al. (1986) have demonstrated enhanced pituitary sensitivity to the negative-feedback effects of glucocorticoids on further ACTH release. This suggested that the increased free concentrations of corticosterone acting via GR could result in the tonic suppression of total circulating concentrations of these hormones. The current work emphasized the importance of testing various aspects of HPA function since the measurement of absolute concentrations of cortisol in the absence of information about plasma CBG concentrations would have incorrectly supported the existence hyporesponsive periods. In addition, the effect of decreased CBG on corticosterone signalling to GR in neonates performed in the present study and by Viau et al. (1996), demonstrated the effects of reduced CBG on increasing the amount of GR translocated by binding to a relatively lower total corticosterone level relative to adult animals.

The primary purpose of the work described in Chapter 5 was to attempt to mimic the effects of neonatal handling on hippocampal GR and HPA function using exogenous quipazine. This was unsuccessful. Neonatal quipazine treatments did not alter hippocampal GR concentrations; however, it did result in a reduction in plasma ACTH response to restraint which was identical to that seen in handled rats. In order to explain how this occurred in the absence of changes in hippocampal GR, mechanisms of central drive on CRH were examined: specifically the serotonergic system. It was found that augmenting 5-HT in neonates resulted in an increase in 5-HT transporter concentrations in the PVN. This is believed to represent increased uptake of (released) 5-HT in this structure resulting in a diminution of 5-HT signalling to the parvocellular (CRH-containing) neurons of the PVN. This decreased duration of 5-HT presence in the synaptic cleft could result in a decrease in CRH release during stress responses. The work described in this chapter is paralleled by that performed on the augmentation of 5-HT signalling using antidepressant treatments in neonates, which resulted in reduced hypothalamic 5-HT content (Feenstra et al. 1996).

The use of neonatal antidepressant treatment has been used to develop an animal model of endogenous depression. Rats which were treated with antidepressants during the neonatal period show many of the behavioural markers of depression as adults (Hilakivi et al. 1987, Vogel et al. 1990). It is hypothesized that the hypothalamus is the key structure mediating the effects of decreased 5-HT on endogenous depression in this animal model: specifically via increased 5-HT transporter concentrations. The results described in Chapter 5 reflected the extreme complexity of the 5-HT system in the brain in that subtle differences in receptor activation can result in dramatic differences in adults. For example, the observation of adults with reduced drive on CRH release via activation of 5-HT$_{1,2 \text{ and } 3}$ receptor subtypes during early life. This is contrasted by the stress-hyporesponsiveness of the neonatally handled rat which resulted from the hypothesized activation of 5-HT$_7$ receptor subtypes (Laplante et al. 1997).

The same paradigm of augmentation of the 5-HT signal was tested in neonatal pigs and was found to elicit no effect on central GR concentrations and no difference in cortisol response to an injection stressor in neonatal pigs. This paralleled the results in rats and it is of interest whether the differences found in PVN 5-HT transporter concentrations would also be found in pigs. However, this remains unknown. It is not known whether pigs treated neonatally with quipazine would show reduced serotonergic drive on CRH release and whether these pigs would in fact, show endogenous depressive patterns of behaviour. The use of rats as a model for human brain function is the foundation for basic research in the medical field and if similar effects can be demonstrated in a "higher" species, with regards to cortical development, such as the pig, this would lend credence to the applicability of the rat as a model for functioning in humans.

A parallel effect of 5-HT signalling augmentation on eliciting HPA activity in pigs was found. In the rat, 5-HT is known to induce CRH release (Johnson et al. 1992) and 5-HT release occurs upon exposure to stressors (Shimizu et al. 1992). The central serotonergic system may therefore represent another mechanism involved in individual differences in stress responses as the current work has shown in the rat. Whether or not this can be a source of variation in the pig is unknown, however the involvement of 5-HT in activation of the HPA in this species has been demonstrated in the current work.

Chapter 6

The experiments described in this chapter were carried out to test the effects of neonatal handling in pigs, based on the evidence provided by Hemsworth et al. (1986;1992), that a sensitive period exists during development in pigs in which neonatal handling influenced future behaviour. Neonatal handling did not produce the same changes in HPA functions in the pig as it does in the rat; specifically stress responses were not attenuated and hippocampal GR concentrations were not increased. Neonatal handling did however cause permanent changes in HPA function in pigs, with increased CBC and resultant decreased free basal cortisol concentrations. Peak ACTH responses to stressors were elevated, possibly as a consequence of a decreased tonic negative-feedback effect on ACTH release, possibly via alterations in CRH release, resulting from the lower basal free cortisol concentrations. We also found that neonatally handled boars could be distinguished behaviourally from nonhandled boars by greater exploration of the inner squares in an open field test. This data supported the hypothesis of a sensitive period postnatally for HPA development in the pig.

In the rat the alterations in interactions between the mother and the offspring has been shown to be key to the neonatal handling effect. Mothers of handled litters licked and groomed their pups with a greater frequency than mothers of nonhandled litters (Weaver and Meaney 1997). In fact, Lui et al. (1997) have shown that a continuum occurred in a random sample of rats where mothers who exhibited high concentrations of licking and grooming and arched-back nursing had offspring who as adults resembled neonatally-handled rats. This was manifested by elevated hippocampal GR and decreased ACTH and corticosterone responses to stressors compared to offspring from mothers who exhibited low licking and grooming and arched back-nursing behaviours. This difference in maternal behaviour is therefore believed to be responsible for individual variation in stress responses in rats. Pigs do not have such intense tactile interactions with their young so it still remains a question as to what is the driving force for the changes in HPA function that have been found in neonatally-handled pigs. In addition, whether a sample of pigs would show that these differences exist in pigs normally due to intrinsic differences in the early neonatal environment remains to be determined. It was noticed that the mothers of the handled litters were very anxious upon separation from their young and tended to nurse the litters immediately upon return to the farrowing crate, regardless of whether they had nursed immediately prior to removal from the crate. This suggests that nutritional factors in the neonate may play a role in the handling effect in pigs in that increases in nursing bouts may occur.

It is unknown whether there are qualitative differences in mother-infant interactions that are important for development in pigs. Sows have been shown to exhibit maternal behaviours such as lying in a lateral recumbent or sternal position with higher plasma cortisol levels associated with more sternal than lateral recumbency (de Passille et al. 1990). Changes in maternal behaviour could be induced by vocalizations between the neonatal pigs and the sows during the separation as these calls are known to communicate information about hunger and temperature (Weary et al. 1997). It is not known whether maternal behaviours mentioned above are altered in sows of the handled litters compared to sows of nonhandled litters, and whether they arise from vocal cross-talk between the litters and the sows, however, it presents and interesting avenue to explore.

Alternatively the difference may result from changes in the neonate itself in that increased sensory stimulation (placed in a novel environment during handling) or fluctuations in plasma hormone concentrations in response to separation may be involved; however, this remains speculative at this time.

An important finding that emerged from the current work was that the effects of neonatal handling in pigs was permanent, in that differences in HPA function were present at 7 months of age. The increased CBC found in handled pigs suggests one possible hypothesis of an increased CBG production occurring in response to handling. The genes encoding the rat and human CBG have been cloned and several potential cis regulatory elements have been identified (Underhill and Hammond 1989;1995). Regulation of transcriptional activity of the promoter has not yet been uncovered. Differences in CBG binding capacity can also be attributed to other factors besides increased protein production. The difference may be due to changes in binding capacity or affinity rather than actual increases in the level of the protein. Differences could also arise from changes in CBG protein turnover rates or due to effects on transcription, mRNA stability, or translation.

An alternative hypothesis would be that the changes in CBG in handled pigs occurred in response to the changes in HPA function such as the decrease in total cortisol concentrations exhibited in these pigs. Higher concentrations of corticosterone inhibited the production of CBG in the rat (Dallman et al. 1987) and it may be that decreases in glucocorticoids will act in a converse manner and result in increased CBG. The mechanisms of action and underlying causes of the effects of neonatal handling on future HPA function in pigs, requires more research before it will be elucidated.

Chapters 7 and 8

The experiments described in these chapters focused our attention on genetic factors influencing HPA function; specifically the presence of the mutated sRyR known to cause PSS. In Chapter 7 the differences in HPA between heterozygous and wild-type pigs was described. It was found that heterozygous pigs had lower basal plasma cortisol and ACTH concentrations with no difference in peak responses or return to baseline upon stressor application. This data was supported by the higher incidence of PSE meat in these animals and therefore the presence of PSE was accompanied by differential basal activity of the HPA. The origin of these differences is not known; however, it was hypothesized that differences in central calcium balance may occur resulting in perturbations of neuronal function.

Adeola et al. (1995) have shown that hippocampal 5-HT concentrations were lower in heterozygous pigs compared to wild-type pigs. Evidence exists for increasing calcium content in medium inhibiting CRH release in vitro in hypothalamic cultures (Jones et al 1982). In the current work, it was shown that the sRyR was expressed in the porcine hippocampus and that reduced calsequestrin concentrations were also seen in this structure in heterozygous pigs. This is believed to be indicative of the expression of mutated sRyR. The functional correlate of the reduced CS is not known and as was discussed in Chapter 8, it may simply relate to the absolute concentrations of sRyR in mutated pigs. It remains to be determined whether hippocampal sRyR expression is decreased in heterozygous pigs.

Central GR and MR

An initial aim in this thesis was to examine the role of GR in individual differences in HPA function in pigs. Neither neonatal 5-HT signal augmentation, neonatal handling, or the presence of mutated sRyR altered central GR expression in pigs. Correlations between pituitary GR and cortisol concentrations 1 hour post-dexamethasone injection were found across heterozygous and wild-type pigs. It can be concluded that the pituitary GR functions in a negative-feedback loop in pigs under conditions of high concentrations of dexamethasone administration. In the hippocampus GR was correlated to peak ACTH concentrations in the morning and, therefore, it is hypothesized that hippocampal GRs are involved in controlling peak ACTH concentrations, possibly via CRH release, in a similar way to the mechanism found in the rat. Similarities appeared to exist between the pig and rat in the role of GR in HPA function, in that it appeared to be involved in negative feedback during circadian peaks and high concentrations of dexamethasone. The brain sites were also similar between the two species in that the site of action of dexamethasone in both the rat and pig are the pituitary, and the control of circadian peaks was affected by the hippocampal population of GR in both species. MR receptors were not examined in this thesis due to methodological difficulties. To date, a commercially available MR antibody, which cross-reacts in pigs, is not available and the porcine MR mRNA sequence is also not known. The changes reported in this thesis for the effects of neonatal handling and PSS all involve basal hormone concentrations during the nadir period, and it is MR receptors that are involved in controlling these concentrations in the rat. Apo-sus rats had higher hippocampal MR measured with a receptor binding assay in ADX rats (Rots et al. 1993). Reductions of MR are associated with elevations in nadir plasma (Ratka et al. 1989, Rots et al. 1996). In the apo-sus rats one would expect the elevated MRs to result in reduced ACTH which was not the case. These rats resembled the neonatally-handled pig in that they exhibited elevated CBG, reduced free cortisol and increased peak ACTH concentrations. It may be that neonatally-handled pigs also exhibit changes in central MR receptors.

In the current work two animal models of altered basal HPA function have been studied: the neonatally handled pigs and the heterozygous mutated sRyR pigs. Their differences in basal HPA function are different in that handled pigs had either no difference, or possible elevations in peak ACTH, with reduced total cortisol concentrations whilst heterozygous pigs exhibited the more predictable relationship of reduced nadir ACTH along with reduced nadir cortisol. It would be interesting to examine differences in central MR receptors between these two types of pigs in order to differentiate the role of MRs in controlling nadir ACTH concentrations in the presence of reduced cortisol.

MR receptors are not the only factor controlling basal HPA function. Small amounts of exogenous corticosterone administered in the morning resulted in compensatory decreases in PM corticosterone concentrations in the rat (Akana et al. 1992). This example shows the importance of multiple time point sampling across the entire circadian cycle in order to elucidate the source of differences in nadir HPA function. In the current experiment there were not sufficient time points during peak circadian activity in both the handling experiment and the PSS animal work. This would be required in order to determine whether differences in peak activity are influencing nadir activity. Based on the apparent lack of difference in central GR concentrations one would hypothesize that differences in peak concentrations would not occur, however differences in central drive (such as found with our rats given quipazine as pups) may exist which affect the peak HPA function. In addition to an examination of central MRs in neonatally handled and heterozygous pigs, a complete circadian profile of plasma ACTH and cortisol would also be required in order to attempt to elucidate the mechanism of changes in basal nadir hormone concentrations.

Remote sampling technique

The work with the PSS and handled pigs also exemplifies the importance of remote sampling when examining basal HPA function. If the samples had not been obtained remotely no differences would have been found in the basal cortisol concentrations in both experiments as a mild stress response would occur upon sampling and in both experiments peak stress-induced cortisol concentrations did not differ. Our use of hourly sampling for basal hormone determination has previously been validated (Barnett et al. 1981) in that sampling at hourly intervals were as accurate as 10 minute intervals due to the relative lack of pulsatility of release during nadir periods of cortisol release. In addition, information about basal concentrations allowed the determination of what background functioning level stress responses are occurring. In the neonatal handling experiment and the experiment with the PSS heterozygous pigs, the cortisol response to a stressor applied in the afternoon did not differ from controls. However, this response could represent a greater increment in cortisol above the relatively lower basal concentrations at that time. Most experiments deal with the effects of absolute increases in glucocorticoids induced by stressors, however there may be important effects of differential increments above basal concentrations on physiological functioning. Such a hypothesis remains to be elucidated; however, both the neonatally-handled pigs and the PSS pigs could be valid models to use in testing this hypothesis.

Basal HPA function and body weights

Of interest was the relative reductions in growth rates and fat content in pigs that had reduced basal nadir cortisol concentrations. Numerous hypothese could be put forward to explain this and in Chapter 6 the bitonic response of body weight and the linear response of fatness to increasing corticosterone concentrations in rats has been discussed. Whether this relationship holds true in pigs remains to be determined but the current work provides preliminary evidence that this is, in fact, the case. In adult male rats experiencing chronic social stress over 14 days, significant elevations in basal corticosterone were detected in subordinate rats (Blanchard et al. 1995). These animals showed significantly greater weight loss than dominant rats compared to control rats not housed in the "stressful" social environment of the visible burrow system (Blanchard et al. 1995). This would provide an animal which is at the other end of the spectrum where elevations in corticosterone are now suppressing weight gain. This is the more conventional situation in experiments examining the effects of cortisol or corticosterone on growth.

It is important not to become "HPA-centric", since measurements of plasma insulin, growth hormone, IGF, and glucose (amongst numerous others) are important in providing indications of how the cortisol concentrations are related to hormones involved in anabolic growth.

Brindley (1992) has described the synergism between elevated cortisol and insulin as a signal for energy deposition and it would be interesting to know whether the treatments used in this thesis resulted in cortisol: insulin ratios which lead to decreased fat composition in neonatally-handled and heterozygous boars. It can be concluded from the experiments described in this thesis that the interactions between cortisol and growth rates are more complex and that reductions in basal cortisol concentrations do not always lead to an enhancement of growth rates.

Adrenal insensitivity

In the present studies with augmented 5-HT in neonatal rats, neonatal handling in pigs, and the effects of PSS on HPA function evidence has been obtained for variation in adrenal function, therefore indicating that this organ is an important source of individual variation in HPA function. In Chapter 5 a hyper-responsivity of the adrenal gland to reduced ACTH in quipazine-treated rats in response to stressors has been demonstrated. In contrast, neonatally-handled pigs had increased ACTH responses to stressors, in the presence of no differences in cortisol responses, thus indicating a relatively hyporesponsive adrenal gland. In Chapter 7 it has been suggested that heterozygous pigs show a slower adrenal response to dexamethasone suppression of ACTH. The data indicated that in all situations differences may be present in the adrenal gland which remains to be determined. Ratios of ACTH:cortisol could be calculated: however, a proper assessment of adrenal function requires examining cortisol responses to a controlled amount of ACTH injected before conclusions can be made. An alternate hypothesis is that the metabolism of corticosterone or cortisol may differ as a function of treatment with increased half-life of these molecules being interpreted as an adrenal hyper-responsivity or sluggish response to ACTH. With this line of reasoning handled pigs could then be interpreted as having a shorter cortisol half-life with absolute concentrations of cortisol being enhanced in response to stressors at the same time as increased degradation of cortisol was occurring. It remains to be determined whether the treatments tested in this thesis result in changes in adrenal function or cortisol metabolism.

Concluding statements

In summary, the objectives of this thesis were to examine sources of individual variation in HPA function in pigs and possible factors involved in generating these differences. It has been demonstrated that early neonatal environment can play an important role in determining future HPA function in swine. In addition, the genetic basis of PSS also resulted in alterations in HPA function. Information has been provided on the role of central GR in HPA function in pigs; however, none of the treatments used caused alteration of this receptor in any region examined. This thesis has described unique HPA profiles depending on early experiential factors or treatments, and genetic composition of animals. The results presented in this thesis have stimulated numerous future research plans in order to further characterize individual differences in HPA function in pigs.

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